UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE QUÍMICA E BIOQUÍMICA

Retinoic Acid in Enteric Lymphoid Organogenesis

Francisca Monjardino Ferreira de Almeida

Mestrado em Bioquímica

2010

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE QUÍMICA E BIOQUÍMICA

Retinoic Acid in Enteric Lymphoid Organogenesis

Francisca Monjardino Ferreira de Almeida

Dissertação orientada por Doutor Henrique Veiga Fernandes

Orientador Interno: Prof. Doutora Maria Margarida Telhada

Mestrado em Bioquímica

2010

"Keep your dreams alive. Understand to achieve anything requires faith and belief in yourself, vision, hard work, determination, and dedication. Remember all things are possible for those who believe."

Gail Devers

Acknowledgments

Ao Henrique por me ter escolhido no meio de uma pilha de CVs! Por toda a exigência e disciplina em tudo o que está envolvido.

À Prof. Margarida pelo seu entusiasmo contagiante pela Imunologia e pelos seus braços abertos pronta para ajudar.

À Lara por tudo e mais alguma coisa… Desde as imensas horas de confocal, às fotos ao fim-de-semana até às gargalhadas contagiantes, o imenso apoio e as festinhas infantis!!

À Manuela e ao Gonçalo por terem cedido os seus resultados, permitindo-me assim ter um história mais completa para contar.

Ao Afonso por todo o apoio e crítica mesmo quando não era a melhor altura. Sem esquecer as piadas mais memoráveis de toda a UIB!

Ao Diogo por não ter papas na língua como eu; Inês por alegrar o nosso espaço com os melhores desenhos e cores; Sílvia por partilhar o desespero bem como o belo do sotaque açoriano.

A todos os que fazem ou fizeram parte da equipa da UIB, bem como do IMM no geral.

A toda a equipa da Unidade de Citometria de Fluxo, à da Animal House bem como à da Gene expression do IGC.

À Marta por me ter cativado nas aulas de Inglês levando-me a escolher imunologia e IMM como local de começo de carreira. Por todos os momentos em que me ofereceu o ombro…

À Ana, Isabel, Íris e Julie por me terem aturado em momentos de crise e por todos os conselhos e mais alguns que serão imensamente úteis para o futuro.

À minha Mãe por ser melga e me estar sempre a telefonar nas horas mais inoportunas.

Ao meu Tio Zé, pai mais do que substituto, e Rita por me terem ajudado a tornar naquilo que sou hoje. A toda a Família pelo apoio e confiança que depositaram em mim.

À minha "nova família", em especial à Teresa, por me terem adoptado e apoiado (e alimentado!) quando os de sangue verdadeiro se encontravam longe.

À Joana e Catarina, pelos belos jantares e idas ao cinema que foram essenciais para a descontracção em momentos mais stressantes.

Ao Nuno por todas as conversas telefónicas a horas inimagináveis.

Ao André… Pela inigualável ajuda em absolutamente TUDO. Pelo facto de estar SEMPRE lá e ter acreditado mais em mim do que eu própria.

RESUMO

O desenvolvimento embrionário é um período crítico da vida do indivíduo durante o qual são formados os sistemas biológicos essenciais à sua sobrevivência. Exemplo disso é o sistema linfóide dos mamíferos, sistema este que consiste numa rede de órgãos linfóides que são classificados em primários ou secundários. É de notar que ao longo da vida adulta outras estruturas linfóides denominadas "terciárias" podem ser formadas, o que acontece no caso de certas inflamações crónicas ou infecções persistentes.

Enquanto os órgãos linfóides primários, tais como a medula óssea e o timo, são locais privilegiados onde ocorre o desenvolvimento das células hematopoiéticas; os órgãos linfóides secundários formam uma rede ao longo do corpo onde residem linhas celulares hematopoiéticas maduras. Os órgãos linfóides secundários incluem, entre outros, os nódulos linfáticos e as placas de Peyer. Estas estruturas são cruciais na iniciação de respostas imunes uma vez que providenciam as condições ideais que levam à activação, diferenciação e homeostasia dos linfócitos. Para o correcto desenvolvimento destes órgãos é necessária a expressão de diversos genes bem como a interacção entre diferentes tipos celulares.

Durante o desenvolvimento dos nódulos linfáticos é necessária a interacção de células hematopoiéticas denominadas células indutoras do Tecido Linfóide (LTi) e de células mesenquimais designadas células organizadoras do Tecido Linfóide (LTo). Esta interacção celular conduz à maturação das células mesenquimais ou de estroma que por sua vez resulta na atracção de células LTi adicionais. Este "feedback" positivo resulta na formação do primórdio do nódulo linfático.

O desenvolvimento das Placas de Peyer (PP) está bem caracterizado e, à semelhança dos nódulos linfáticos, requer interacções entre células hematopoiéticas e de estroma. No entanto, para além das células LTi e LTo, um outro tipo de células hematopoiéticas, denominado células iniciadoras do Tecido Linfóide (LTin) estão envolvidas neste tipo de interacções. O modelo correntemente proposto para o desenvolvimento das PP postula que as células LTin interagem inicialmente com as células LTo através do eixo de sinalização RET/ARTN. Este sinal inicial resultaria então na maturação inicial das células do estroma e consequente produção de quimiocinas que, por sua vez, atrairiam células LTi formando-se assim o cluster celular que está na origem do primórdio do órgão.

Apesar dos paralelos existentes entre o desenvolvimento dos nódulos linfáticos e das PP, estas estruturas não utilizam exactamente os mesmos eixos de sinalização. No caso dos nódulos linfáticos as mais importantes vias de sinalização são TRANCE e Linfotoxina β (LTβ). Inicialmente a interacção entre os dois tipos celulares é feita através da expressão de TRANCE

pelas células de estroma e do seu receptor pelas células LTi. Aquando desta interacção, ocorre a indução da expressão de LTαβ na superfície das células LTi. Por sua vez as células LTo são activadas através do receptor de LTβ expresso na superfície destas últimas. Esta activação induz a produção das quimiocinas CXCL12 e CXCL13 atraindo assim células LTi adicionais. No caso das placas de Peyer as vias de sinalização mais importantes são RET, IL-7R e LTβ. Pensa-se que é através da via de sinalização RET que ocorre a expressão de LTβ nas LTin, expressão esta que permite que ocorra a sua interacção com as células de estroma. À semelhança do que acontece nos nódulos linfáticos, esta interacção de ligando/receptor irá originar um "feedback" positivo, atraindo assim as células LTi para o local do cluster. Estas células por sua vez, através da sinalização dada pelo IL-7R, irão expressar LTβ como as células LTin e interagir finalmente com as células LTo.

A análise de animais deficientes em certas vias de sinalização reforça também a ideia que o desenvolvimento dos nódulos linfáticos e das placas de Peyer não obedece exactamente aos mesmos princípios. A título de exemplo, ratinhos deficientes em TRANCE não possuem nódulos linfáticos uma vez que a maturação das células de estroma é deficiente, no entanto possuem placas de Peyer normais. Por outro lado, deficiências no receptor da quimiocina IL7 resultam no desenvolvimento de apenas alguns nódulos linfáticos, demonstrado que esta via é importante, mas não crucial, para o desenvolvimento destas estruturas linfáticas. Contudo, estes animais são totalmente desprovidos de placas de Peyer, provando que esta via é essencial para o desenvolvimento deste tipo de estruturas entéricas. É ainda de notar que a via de sinalização RET é apenas essencial para o desenvolvimento de placas de Peyer . Ratinhos deficientes nesta tirosina quinase apesar de desenvolverem nódulos linfáticos são totalmente desprovidos de placas de Peyer.

O processo de desenvolvimento embrionário requer no sentido mais lato várias vias de sinalização, sendo exemplo disso a do ácido retinóico (RA). Recentemente, o ácido retinóico surgiu como uma das moléculas importantes no desenvolvimento dos nódulos linfáticos. Van Pavert *et al.* descreveram que o RA produzido por células nervosas, adjacentes a células de estroma, induzia nestas últimas a produção da quimiocina CXCL13. Desta forma, esta molécula parece estar envolvida no despoletar de uma retroacção positiva que origina o primórdio dos nódulos linfáticos. Uma vez que o ácido retinóico parece ser importante no desenvolvimento dos nódulos linfáticos, pretendemos determinar se esta via de sinalização é também importante para o desenvolvimento das estruturas linfóides entéricas, nomeadamente das Placas de Peyer.

Com este intuito, inicialmente utilizámos ratinhos geneticamente modificados que permitem a visualização, análise e manipulação *ex vivo* de todas as subpopulações hematopoiéticas

durante a organogénese linfóide. A utilização destes modelos permite a micro dissecção de estruturas linfóides secundárias no embrião e a purificação *ex vivo* de populações hematopoiéticas e mesenquimais genuínas em diferentes estádios de desenvolvimento. Em paralelo, através de estudos imunocitoquímicos e genéticos, verificámos que o intestino embrionário alberga não apenas células que exprimen RALDH1, enzima importante na síntese de RA, bem como células alvo de RA. Tendo em conta estes modelos e os resultados assim obtidos prosseguimos com os objectivos propostos.

De modo a compreender a influência do RA na formação das estruturas linfóides entéricas utilizámos culturas de intestinos embrionários nas quais é possível colocar micro-esferas de agarose impregnadas com ácido retinóico. Neste sistema observámos que o RA induz a formação de agregados de células hematopoiéticas que por sua vez induzem a expressão de VCAM-1 em células de estroma, formando assim estruturas linfóides embrionárias atópicas. É de notar que a composição celular dos agregados celulares formados pela sinalização RA é idêntica à composição das estruturas formadas pela sinalização via RET, isto é, células LTi e LTin.

Relevante também é a correlação destas duas vias de sinalização, que foi possível estudar com base em culturas de intestinos embrionários onde se bloqueou a via de sinalização do ácido retinóico, fornecendo simultaneamente a sinalização por RET. Este bloqueio diminuiu significativamente a eficiência de agregação celular pela sinalização RET. Por outro lado, na ausência de RET, a eficiência de agregação por RA não foi modificada. Estes resultados sugerem que a via de sinalização por RA actua independentemente de RET e desempenhara a sua função maioritariamente a montante de RET. Adicionalmente verificámos que, o RA não aparenta estar envolvido na motilidade das células hematopoiéticas durante a organogénese entérica linfóide.

O ácido retinóico está directamente interligado com a produção de quimiocinas durante o desenvolvimento dos nódulos linfáticos. Uma vez que esta molécula parece ter um papel semelhante no desenvolvimento das PP, é plausível que estas quimiocinas também estejam envolvidas na sinalização de RA durante o desenvolvimento destas estruturas. Com este pressuposto em mente, foram analisadas por reacção quantitativa de polimerase em cadeia (qPCR) amostras celulares de intestino fetal estimuladas com ácido retinóico durante 24h. Esta análise revelou que nem CXCL12 nem CXCL13 estavam sobreexpressas após estimulação. De forma a confirmar este resultado foram feitas culturas de intestinos embrionários onde foi fornecido o sinal de ácido retinóico e bloqueadas as quimiocinas em questão. Os resultados obtidos vieram confirmar a conclusão anterior, demonstrando que CXCL12 e 13 não são importantes para a sinalização de RA no desenvolvimento das placas de Peyer. Contudo, uma

vez que outras quimiocinas podem estar envolvidas no desenvolvimento destas estruturas, foram feitos ensaios de expressão genética a partir de amostras celulares entéricas estimuladas com ácido retinóico. Estes estudos revelaram que a via de sinalização pelo ácido retinóico resulta numa assinatura genética que difere da previamente descrita durante o desenvolvimento dos nódulos linfáticos, apontando para um possível papel na diferenciação das células hematopoiéticas.

Em conjunto, estes resultados apontam para um papel do ácido retinóico no desenvolvimento das placas de Peyer. O ácido retinóico emerge então como uma nova molécula importante na organogénese destas estruturas.

Palavras chave: Ácido retinóico, Placas de Peyer, sinalização por RET, órgãos linfóides secundários, organogénese.

ABSTRACT

Secondary Lymphoid Organs (SLOs), such as Lymph Nodes (LN) and Peyer's Patches (PP), are crucial structures for the initiation of immune responses. Several molecules are required for the development of these structures, namely Lymphotoxin and several chemokines, while the tyrosine kinase RET has been shown to play a key role in PP organogenesis. Retinoic Acid (RA) has recently emerged as an important player during LN development through induction of CXCL13 expression. Despite many similarities, LN and PP development differ in several aspects.

Here we show that RA specifically induces clustering of enteric haematopoietic cells, and is sufficient to induce the maturation of sessile mesenchymal cells. Interestingly, both RET and RA induced clusters are similarly composed by LTi and LTin cells. This finding suggested that RA and RET signalling could participate in the same events during PP formation. However, haematopoietic clustering induced by ARTN was only marginally affected by RA signalling block and absence of RET signalling did not impact on RA mediated clustering. Altogether, these results suggest that RA acts independently and mainly precedes RET signals.

Furthermore, and contrary to LN development, we show that chemokines such as CXCL12 and 13 are not upregulated in gut samples stimulated with RA, neither affect clustering efficiency by RA. Altogether these results suggest that during PP development RET and RA signals act mostly independently of each other and that enteric RA signalling leads to a genetic signature that differs from what was previously described during LN development.

RA emerges as a novel molecule in PP organogenesis. We suggest that this pathway may be involved with other signalling pathways in the processes that underlie enteric lymphoid organ formation and haematopoietic differentiation. These hypotheses will be further discussed taking advantage of both *in vitro* and *in vivo* approaches.

Keywords: Retinoic acid signalling, Peyer's Patches, Ret signalling, SLOs, organogenesis

TABLE OF CONTENTS

I. INTRODUCTION

1. GENERAL ASPECTS

The mammalian lymphoid system consists of a network of lymphoid organs that are classified as either primary or secondary lymphoid organs. Whereas primary lymphoid organs, such as the bone marrow and the thymus, are major sites where haematopoiesis occurs, secondary lymphoid organs (SLOs) form a network throughout the body and harbour essentially mature haematopoietic cell lineages. SLOs include the spleen, lymph nodes (LN) and Peyer's Patches (PP). Importantly, there are several mucosal-associated lymphoid tissues (MALT) that are determinant for mucosal immunity. These include the nasal-associated lymphoid tissue (NALT), bronchial-associated lymphoid tissue (BALT), gut-associated lymphoid tissue (GALT) and other less-prominent organized clusters of lymphoid cells associated with the genitourinary tract[1, 2]. Several structures contribute for the GALT and they include the Peyer's Patches (PP), the Crypto Patches and Isolated Lymphoid Follicles, and the Intraepithelial Lymphocytes [3].

SLO development occurs during embryonic life, but in some pathologies, such as persistent infection or chronic inflammation, "tertiary" lymphoid structures can also be formed in adulthood [4].

2. SECONDARY LYMPHOID ORGANS

Secondary lymphoid tissues differ in their detailed structures but share some common features. Each consist of a supporting stromal matrix that provides an organizing framework for T and B lymphocytes, antigen-transporting and antigen-presenting cells, as well as other regulatory cells [2]. These tissues also possess a massive vascular supply that allows them to perform their functions [2]. However, despite sharing basic structural features and functions, secondary lymphoid organs differ in their localization and development. SLOs develop during embryogenesis or in the early postnatal period at predetermined sites throughout the body as a result of complex cellular interactions [4].

2.1. Function of Secondary Lymphoid Organs

Secondary lymphoid organs are located at strategic sites in the body, allowing for rapid recognition of foreign antigens and microorganisms. These organs have specialized structures and microenvironments that contribute for a rapid control of potential infections since they promote interactions of immune cells that rapidly mount an appropriate immune response [4].

After birth, SLOs are colonised, among others, by lymphocytes and antigen presenting cells. These cell types are organized in a manner that optimizes cellular interactions which are determinant for efficient pathogen recognition and elimination. Thus, SLOs are crucial in the

initiation of immune responses since they provide the conditions that lead to lymphocyte activation, differentiation and homeostasis [1].

Lymph nodes and lymphatic vessels, from which the former arise as out-pouches, are part of the lymphatic system. These structures are generally localized at sites of vascular junctions such as inguinal and axillary regions and are divided into two cell compartments, the B and the T cell area [2]. LNs recruit newly produced lymphocytes from the blood and attract activated antigen-bearing antigen-presenting cells from peripheral organs. This large influx of different cell types into the LN allows for rare specific naïve T or B lymphocytes to encounter their cognate antigen. Thus, lymphocyte recirculation throughout the LN network contributes for more efficient immune response [4]

Peyer's patches are the major SLOs of the gut-associated lymphoid tissue. PPs are specifically located in the antimesenteric wall of the small intestine [2]. Although all mammals have PP, they vary considerably in distribution and number in different species; adult mice have 6 to 12 while humans can have up to 240 PPs [2, 5-7]. PP are covered, on the luminal side of the intestine, by a specialized epithelial layer that constitutes the follicle-associated epithelium (FAE). M cells, which are components of FAE, uptake antigens from the gut lumen, and have also been shown to transport particles as large as intact microorganisms [2]. After sampling, antigens are processed in dendritic cells and presented to lymphocytes [1, 2, 4, 8]. A significant part of IgA B cells are generated in a T cell-dependent manner in the PPs and this process partly depends on follicular CD4 helper T cells. In fact, key markers of Ig class switch, such as AID [9] and germline alpha transcripts [10] are highly expressed in PP when compared to mesenteric lymph nodes [11]. Thus, PPs are major sites for the induction of IgA antigen specific responses.

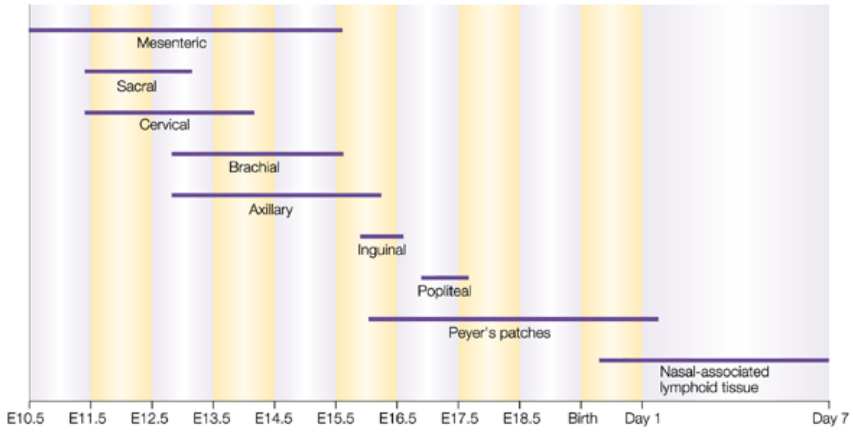


Figure 1. **Chronological development of various lymphoid organs**, including several types of lymph nodes, Peyer's patches and NALT (adapted from[12]).

2.2. Development of Secondary Lymphoid Organs

The early post-implantation phases of development are characterised by the formation of the extra-embryonic structure placenta, the yolk sac, the allantois and the amminon. Collectively, the functions of these structures sustain the viability of the embryo by primarily acting as the fetal-maternal interface through which nourishment and growth promoting factors reach the embryo. Organogenesis begins with the assembly of progenitor tissues into organ primordia. Subsequent triggering by inducing tissues results in the initiation phase, which is characterised by active cell proliferation that builds up a critical tissue mass for morphogenesis and specification of diverse cell types. In the aggregation phase, through cell clustering and outgrowth of the primordium, organ rudiments are finally formed [13, 14].

The earliest event in LN development is the formation of lymph sacs [15]. These structures result from endothelial cell budding from large veins during embryogenesis. Lymph sacs are then involved by the surrounding connective tissue and cells from haematopoietic origin. In mice, lymph sacs start to form around Embryonic Day 10.5 (E10.5) and this process is completed by E12.5 [15]. Haematopoietic cells in LN primordia develop in the foetal liver and migrate through the blood to sites of anlagen development [16, 17]. The development of LN and PP is not synchronised but sequential from the anterior to the posterior part of the embryo (Figure.1).

Peyer's Patches development initiates at the proximal end of the intestine and subsequently proceeds towards the distal end [12]. This process is clearly defined by three steps. The earliest event is the formation of specific "spots" that co-expressed Vascular-Cell Adhesion Molecule 1 (VCAM-1) and Intercellular-Cell Adhesion Molecule 1 (ICAM-1) at E15.5 (fig.1) [18]. This is followed by the accumulation of haematopoietic cells in these "spots". Finally, the third phase is the appearance of CD3 and B220 positive cells [18].

3. DEVELOPMENT OF SECONDARY LYMPHOID ORGANS

3.1. Lymph Nodes

3.1.1. Cellular Interactions

Lymph nodes are capsulated by lymphatic endothelium and their development occurs concurrently with the process of lymphatic vascularisation [2]. Based on histological observations, different stages of LN development of can be distinguished. The first stage is characterized by the formation of the lymph sacs and it is followed by a second step, which consists in the sprouting of lymphatic vessels from these structures around E10.5 [2]. In the third stage, LN anlagen are formed by the protruding of the connective tissue into these lymph sacs.

So, the early LN anlagen is surrounded by endothelial cells that have characteristics of both lymphatic and blood vascular endothelial cells [4, 19], but the lymphatic vasculature is only complete by E15.5[4]. The colonization of the LN anlagen by haematopoietic cells constitutes the fourth stage [20]. In the fifth and latest stage, the LN continues to expand, the cellular density and leukocyte content continue to increase, and mature lymphocytes migrate into the developing organ and establish a proper cellular microarchitecture [1, 21].

Among the earliest haematopoietic populations colonizing the LN is the $CD45^{pos}CD4^{pos}CD3^{neg}$IL-$7R\alpha^{pos}$c-$kit^{pos}$ cell type. These cells derive from foetal liver precursors and are called Lymphoid Tissue inducer (LTi) cells [16, 17]. Beside this population, others cell types are present in the LN anlagen; mostly of which are IL-$7R\alpha^{pos}$ $CD4^{neg}$ [16].

Resident stromal cells in the primordia are named Lymphoid Tissue organizer (LTo) cells, and are characterised by the expression of the mucosal addressin, MAdCAM-1, VCAM-1 and ICAM-1 [16]. In the LN anlagen LTo cells also expressed the surface TNF family member, TRANCE [16, 21, 22]. LTo cells are present in the endothelium that forms the lymph sac. Accordingly, expression of gp38/podoplanin can be found in the endothelium, which is surrounded by a $perlecan^{pos}$ membrane [23].

It is believed that LTi cells induce LTo maturation. This process induces the production of several signals that at later stages create a positive feedback. As a consequence, circulating haematopoietic cells will be attracted to, and retained within, leading to the formation of the LN anlagen [20, 21]. Thus, the cross-talk between local mesenchymal cells and circulating haematopoietic cells, LTo and LTi, respectively, is the basis for the successful formation of lymph nodes.

3.1.2. Molecular Signalling

During LN development it is believed that TRANCE signalling is on the onset of haematopoietic LTi cells and presumably sessile mesenchymal LTo cells interactions. TRANCE (TNF-related action-induced cytokine, also known as OPGL/ODF/RANKL/TNFSF11), its receptor TRANCE-R (TNF-related action-induced cytokine receptor, also known as OFE/ODFR/RANK), and a critical component of the TRANCE signalling pathway, TRAF6 (TNF receptor-associated factor 6), are all required for LN development as defined by the absence of LNs in mice deficient for these genes[4, 21].

The TRANCE/TRANCE-R signalling axis operates when LTi cells migrate into the LN anlagen. TRANCE is expressed by LTo cells, and its interaction with TRANCE-R expressed by LTi cells, leads to the up-regulation of $LT\alpha_1\beta_2$ on the latter [19, 21]. Accordingly, $TRANCE^{-/-}$ mice do not develop LNs since maturation of stromal cells is deficient [21, 24].

When the number of LTi cells reach a critical threshold, the interaction between haematopoietic and stromal cells via the LTβ/LTβR system leads to the homotypic interaction of LTi cells to form compact clusters. These clusters of LTi cells provide a community effect for subsequent differentiation of LTi cells themselves and LTo cells [21].

In fact, through LTβR expressed by LTo cells, LTi cells promote the differentiation of the latter[23, 24]. Initially, immature mesenchymal cells are ICAM$^-$VCAM$^-$ [23]. Through a yet unknown signal, these cells are primed to give rise to ICAM-1^{int}VCAM-1^{int} gp38/podoplanin^{pos} cells [23]. The latter recruit additional LTi cells since they express IL-7, CCL21 and CXCL13 [16, 24, 25]. Through LTβR engagement, mesenchymal ICAM-1^{int}VCAM-1^{int} cells give rise to a mature population of stromal organizer (LTo) cells, which has the phenotype ICAM-1^{high}VCAM-1^{high}MAdCAM-1^{pos} [23]. It is believed that the combined effect of chemokines and adhesion molecules result in a positive "feedback" loop that ultimately promotes LTi clustering and recruitment of newly emerging haematopoietic cells to the developing LN [23, 24].

IL-7Rα signalling is also important for LN development, although IL-7Rα deficient mice still develop some LNs [26]. Activation of IL-7Rα^{pos} inducer cells elicits signals that are believed to cooperate with LTαβ in the formation of the complete LN structure. IL-7, acting as an alternative signal for TRANCE, only partially restored LN genesis in TRAF6$^{-/-}$ mice. This finding indicates that TRAF6-propagated signals, including those upstream of TRANCE-R, are involved in the formation of the higher order LN micro-architecture [24].

3.2. Peyer's Patches

3.2.1. Cellular Interactions

During PP development different cell types interact with each other resulting on PP formation starting from the anterior to the posterior part of the small intestine [18]. This process is divided in three distinctive steps according to the phenotype and cell types present at the PP primordia [18, 22].

As for lymph node development, the cellular mechanisms implicated in PP development are well characterized, relying on interactions between cells from haematopoietic and mesenchymal origin. Despite the parallels between LN and PP genesis, these processes are not entirely identical and even require differential players.

Emerging embryonic haematopoietic cells, believed to be the progeny of a foetal liver progenitor CD3^{neg}CD4^{neg}cKit^{pos}IL7Rα^{pos}α4β7^{pos} [27, 28], start to colonize the gut at E12.5 [5]. By E15.5 increasing numbers of highly motile haematopoietic cells are found evenly distributed throughout the gut and by E16.5 these cells aggregate to form the PP primordia [5, 18, 22, 29]. Thus, while LN localisation is seemingly determined before the migration of haematopoietic cells to

prospective sites of development, location of PP anlagen does not seem to be strictly pre-determined.

Haematopoietic cells that initially colonise the intestine include $CD4^{pos}CD3^{neg}L7R\alpha^{pos}c\text{-}Kit^{pos}$ Lymphoid Tissue inducer (LTi) cells [18, 22, 29] and a phenotypically distinct population of $CD4^{neg}CD3^{neg}c\text{-}Kit^{pos}IL7R\alpha^{neg}CD11c^{pos}$ Lymphoid Tissue initiator (LTin) cells [5, 30]. Both LTi and LTin aggregate together with mesenchymal origin $VCAM\text{-}1^{pos}ICAM\text{-}1^{pos}$ Lymphoid Tissue organiser (LTo) cells [25, 31] forming the PP primordium [5, 18, 29, 31].

Haematopoietic LTi and LTin cells play important roles in PP development. Hence, in the absence of LTi cells, which occur in *Id2* [32] and *Rorc* [33] deficient mice, PP fail to develop. Moreover, adoptive transfer of LTi cells into neonatal mice with minute numbers of PP was shown to rescue the organogenesis of these structures [34]. In agreement with this idea, increased LTi cell numbers, obtained by IL7 over expression, result in high number of PPs [35]. Concerning LTin cells, selective and partial ablation of $CD11c^{+}$ cells result in impaired PP development, and mice deficient for the tyrosine kinase receptor RET, expressed by LTin cells, do not form PP [5]. Most importantly, the use of the RET ligand ARTN in explants organ cultures of embryonic intestines induced LTin and LTi clustering and up-regulation of VCAM-1 by mesenchymal cells, resulting in ectopic PP primordia [5]. During LN development $CD11c^{+}$ cells were also described in the anlagen [24], however the relationship between LN and enteric $CD11c^{+}$ cells is unclear and it is not know what functions these cells may play in the context of LN genesis.

While most molecules implicated in LN development also play a role in PP formation, some signalling pathways are not equally used in both processes. As an example, while IL7R signal is crucial for PP development, as revealed by $Il7r^{-/-}$ mice [22, 29], brachial, axillary and mesenteric LN develop normally in these animals [12]. Similarly, while in the absence of RET signalling PP fail to form, LN development in $Ret^{-/-}$ mice is seemingly normal [5]. On the other hand, the differential use of molecular pathways is also revealed by mutants of the TRANCE/TRANCE-R signalling axis. While in $Trance^{-/-}$ [21, 36, 37] and $Traf6^{-/-}$ [38] mice LN development is severely compromised, PP development is entirely normal.

At a later stage, lymphocytes start to colonise recently formed PP and $CD3^{pos}$ and $B220^{pos}$ mature lymphocytes start to cluster in distinct zones. This accumulation starts immediately after birth, even though mature lymphocytes are already present in the embryo at E17. The reason behind this colonisation delay might be the fact that the PP vascular network is completed rather late by E18.5.

Hence, although LN and PP genesis display obvious parallels, the differential requirements for their development may also reflect distinct genetic signatures of their respective cellular players.

3.2.2. Molecular Signalling

The first step in PP development is characterized by the VCAM-1 expression by mesenchymal cells [18]. It is believed that this expression results from the interaction between haematopoietic colonising cells and sessile stromal cells.

The first players in this interaction are RET-expressing LTin cells[5]. Engagement of ARTN/GFRα3 with the RET tyrosine kinase receptor, expressed by LTin, results in the maturation of stromal cells as determined by VCAM-1 up-regulation [5, 30]. It is possible that RET signalling leads to LTin direct or indirect LTβ production, resulting in activation of LTβR expressing LTo cells [30]. After this triggering step, the maturation of LTo cells ensures VCAM-1, ICAM-1, and chemokines production, such as CXCL13, CCL21, CCL18 and IL-7 [5, 30].

This triggering step is followed by the attraction of LTi cells by mature LTo cells. It is believed that the chemokines and cytokines produced by LTo cells lead to LTi attraction[5]. LTi cells express several chemokine receptors, such as CXCR5 and CCR7, which respond to the chemokines produced by mature LTo cells and therefore result in a positive feedback loop of LTi attraction and LTo maturation.

LTi cells express IL-7Rα and signal through this receptor by IL-7 (produced by mature LTo cells) leads to up-regulation of LTαβ in their surface [25, 29]. Consequently, interaction between LTi and LTo cells occurs through LTβ engagement, promoting further maturation of stromal cells and expression of several homeostatic chemokines, such as CXCL13 and CCL19 [25, 30]. In addition, the contribution of chemokines, cytokines and adhesion molecules such as VCAM-1 also contribute to PP primordial formation. In fact, mature stromal cells express VCAM-1, while haematopoietic cells express its ligand α4β7 integrin in their surface thus, facilitating their retention near LTo cells[18].

In conclusion, despite the obvious parallels between LN and PP formation, these SLOs exhibit different requirements for their development. Thus, while LN organogenesis requires TRANCE/TRANCE-R signalling, this signal is dispensable for PP development. Moreover, while PP development is IL-7R and RET dependent [18, 21, 22, 39], LN development still occurs in the absence of these molecules.

4. RETINOIC ACID

Retinoic Acid (RA) is a small lipophilic molecule of low molecular weight (300 Da), which is derived from vitamin A (retinol) and it is found in embryos and adult vertebrates. Since animals are unable to produce vitamin A, they obtain this vitamin through their nutrition [40]. The metabolism of this vitamin is a complex process. Firstly, the alcohol form (retinol) enters circulation bound to the retinol-binding protein (RBP4), secreted by the liver [41]. Secondly, retinol enters the cell through a specific receptor, STRA6 [39]. The conversion of the alcohol form into retinyl esters for storage is ensured by CRBP, cellular retinol-binding protein. Consecutive dehydrogenases, such as RALDH (retinaldehyde dehydrogenase), transform retinol into a carboxylic acid, the Retinoic Acid [40, 42]. RA is then released and up-taken by surrounding cells. If the cell uptaking this molecule is a non-target tissue, RA is catabolised by cytochrome P450 enzymes (CYP26 family) into inactive compounds and then excreted [40, 42]. Conversely, if the cell is an RA target, this molecule enters the nucleus, through its specific receptors, Retinoic Acid Receptors (RAR), and binds directly to target genes via one of the two nuclear receptors families. The complex RA-RAR binds to RARE (RA Response Elements) regulating transcription of RA target genes, such as the Hox family (figure 2).

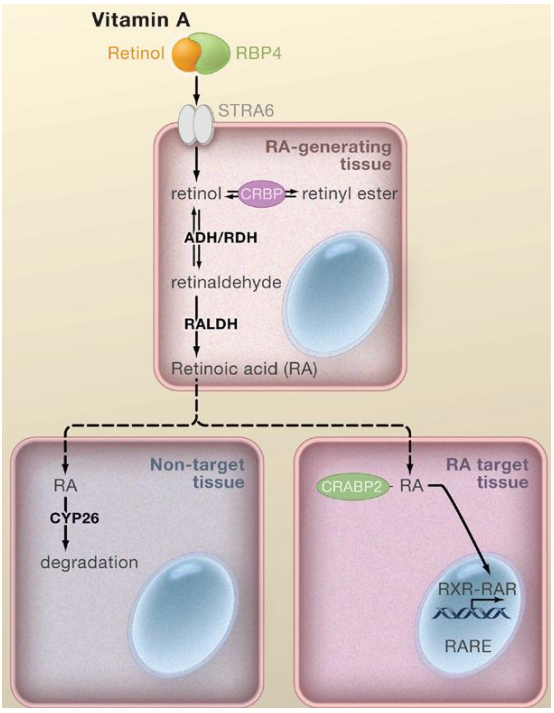


Figure 2. **Retinoic Acid synthesis and signalling** (adapted from [42]). RA is synthesised from Vitamin A through several Dehydrogenases, such as RALDH2. After RA formation, this molecule is uptaken by the surrounding cells. If the cells is a non-target cell, RA is degraded by CYP26. However, if the cell that uptakes RA is a target one, in that case RA migrates to the nucleus and interact with RARE (Retinoic Acid Response Elements).

Through the regulation of the Hox gene family, RA controls the patterning of the antero-posterior embryo axis [40]. This regulation is done through RARE in their regulatory/enhancer activity. RA also regulates other developmental processes, namely body axis extension, neurogenesis or cardiogenesis, through the repression of several growth factor signalling pathways [42].

4.1. Role in Enteric Organogenesis

The balance between vitamin A deficiency and toxicity is a delicate one. Maternal insufficiency of vitamin A during pregnancy results in foetal death and severe congenital malformations, while excess of this vitamin or RA *per se* may induce major alterations in organogenesis because of its teratogenic effect [42, 43].

In early development RA signalling plays many different roles; it provides an instructive signal for the posterior neuroectoderm (hindbrain, spinal cord) and posterior foregut endoderm (pancreas and lungs) and a permissive signal for trunk mesoderm (somites, heart and forelimb) in early development [42, 44]. In particular, Retinoic acid has been implicated in the regulation of many aspects of neuronal development including specification of neuronal fate and stimulation of neurite outgrowth [45, 46]. RA has also been found to promote survival and proliferation of neuronal progenitors in neural crest cell populations [46]. In fact, lack of RALDH2 causes loss of neural crest cell migration and enteric neuron system development [46, 47]. Strikingly, one of the signalling pathways of critical importance in the development of the enteric nervous system is RET [5]. On one hand, Raldh2$^{-/-}$ mice have a deficiency of *Ret*-expressing cells in the stomach and gut wall. On the other hand, the few RET expressing cells are arranged as rudimentary tracts, at the expected location of the vagal nerves. These vagal defects in *Raldh*2$^{-/-}$ embryos lead to a similar gastrointestinal defects, seen in *Ret* or *Gdnf* knockouts mice [46, 48-50]. Even though few RET ^{pos} ENS progenitor cells are detected along the foregut wall at E10.5, they are apparently unable to colonize the gastrointestinal tract, leading to an absence of enteric ganglia [46].

4.2. Function in the lymphoid and Immune Systems

In late 2009, van de Pavert *et al.* showed that RA is involved in the LN organogenesis. The authors proposed that during the formation of lymph node primordia, neuro-derived RA induce CXCL13 expression in mesenchymal cells [51]. This expression is essential for the initial attraction of LTi cells and the whole process determines the location of lymph nodes. The authors further demonstrate that CXCL13 expression is abrogated in mice lacking the RA-synthesizing enzyme RALDH2 and lymph node anlagen are aberrantly formed in RALDH2

knock-out embryos. Since CXCL13 is essential for the initial attraction of LTi cells, and this expression is RA-dependent, the authors concluded that RA is involved in the formation of early anlagen lymph nodes [51].

RA has also multiple functions in the immune system. The production of RA by the RALDH^{pos} DC in PP, mesenteric LN (mLN) and small-intestinal Lamina Propria is critical for both imprinting lymphocytes with gut-homing specificity and differentiation of naive T cells into inducible Foxp3^{pos} regulatory cells [52-56].

Multiple micro-environmental factors in the small intestine contribute to the induction of RALDH in DCs, and their composition appears to influence immune responses in the intestine. DCs and mLN stromal cells deliver positive signals, including RA, that support the induction of gut-homing molecules, such as α4β7-integrin and CCR9. RA expression seems to be involved in the generation of a permissive LN environment inducing gut-homing T cells [53-55]. Besides this, RA has a direct IgA-promoting effect on activated B cells and it also appears to synergize with several other mechanisms that are thought to promote IgA production in the gut [56]

5. AIMS and EXPERIMENTAL MODELS

RA has recently emerged as an important player during lymph node development [51]. However, although other paracrine effects of RA have been described in the immune system, the mechanism by which RA is transferred to adjacent cells leading to the CXCL13 expression is unknown. This fact may also lead to the possibility that this molecule may be involved in other molecular mechanisms during secondary lymphoid organogenesis.

In this project we used genetic, cellular, and molecular approaches in order to understand the role of RA in enteric lymphoid organogenesis.

In order to achieve this, we employed transgenic reporter mouse models and gut explants organ culture in order to follow the responsiveness of haematopoietic cells to RA related molecules. By using this strategy we shed light on what molecules and cell types are involved in RA responses during enteric lymphoid organogenesis.

II. MATERIAL AND METHODS

1. MICE

C57Bl/6J mice were purchased from Harlan™.

Human CD2-GFP transgenic mice [5] and Ret heterozygous mice [49] were bred and maintained at the IMM animal facility. The RARE Lac-Z mice were bread and maintained at Institut de Gén+etique et de Biologie Moléculaire et Cellulaire, CNRS (UMR 7104), Inserm U964, Iniversité de Strasbourg.

All mice were maintained at IMM animal facility. All animal experiments were done in accordance to institutional and national guidelines.

2. EMBRYO ANALYSIS

In order to obtain embryos at different development stages, mice were naturally timed-mated during one night and the presence of vaginal plug was checked the morning after. The plug day was considered as 0.5 days of gestation. In order to obtain CD2-GFP heterozygous embryos, C57BL/6J females were crossed with transgenic human CD2-GFP heterozygous males [5]. CD2-GFP embryos were then collected and screened for GFP expression using a wide-field stereo fluorescent microscope (Zeiss Stereo Lumar. V12 with a Zeiss Neolumar S 0.8x objective). In order to obtain $Ret^{-/-}$ embryos, $Ret^{+/-}$ females were crossed with $Ret^{+/-}$ males. Pregnant females, at the chosen gestational day, were sacrificed and dissected.

2.1. Embryo Dissection and Cell Suspension

After micro-dissection, organs were collected in GIBCO® Dubelco's Modified Eagle Medium (DMEM) (Invitrogen™), supplemented with 2% Foetal Bovine Serum (FBS) (Invitrogen™), 1% Penicilin and Streptomycin (P/S) (Invitrogen™) and 1% Glutamin (Invitrogen™); or, in case of tissue culture, GIBCO® Roswell Park Memorial Institute medium (RPMI) (Invitrogen™), supplemented with 10% Foetal Bovine Serum (FBS) (Invitrogen™), 1% Penicilin and Streptomycin (P/S) (Invitrogen™) and 1% Glutamin (Invitrogen™).

For analysis by flow cytometry (FACS) tissues were brought into single-cell suspension using Collagenase D (5mg/mL; Roche) and DNase I (0.1mg/mL; Roche) in DMEM for 30 min at 37°C, and then passed through 70µm cell strainers (BD Falcon™). Viable cells were counted in a Neubauer hemocytometer using Trypan Blue to assess cell viability [57].

For explant organ cultures GFP-positive intestines were micro-dissected including stomach and caecum.

3. CELL STAINING AND FLOW CYTOMETRY

Antibodies were purchased from eBioscience® and Biolegend®. The antibodies used from eBioscience® were CD45.2 FITC (104), CD4 APC (GK1.5), CD11c (p150/90) PE (N418), ICAM-I (CD54) PE (YN1/1.74), Sca-1 (Ly6A/E) FITC (D7) and CD16/32 (93). The antibodies used from Biolegend® were Podoplanin (gp38) biotin (8.1.1) and MVCAM.A (CD106) PerCP/Cy5.5 (429). Beside these antibodies, streptavidin APC from BD Pharmingen™ was also used. TO-PRO®-3 iodide (T3605) from Invitrogen® was used as a viability dye.

In order to identify embryonic sub-populations, cells from E14.5, E15.5 and E18.5 C57Bl/6J embryos or from hCD2-GFP-C57Bl/6 embryos [5] were analysed by FACS. Antibodies were added to the cell suspensions and incubated for 20' on ice in a rotating device.

Flow citometry results were acquired in BD FACSCanto (Bencton Dickinson) and were analysed using FlowJo software (Tree Star Inc, version 8.8.4)

3.1. Fluorescence-activated cell sorting and RA cell stimulation

Gut cell suspensions were FACS sorted using BD FACSAria (Bencton Dickinson). This was done in order to separate and purify haematopoietic populations from non-haematopoietic populations, $CD45^{pos}$ and $CD45^{neg}$ cells respectively. Cell staining was performed and analysed as previously described. Afterwards, the two populations were stimulated for 6h with 10ng/mL of Albumin bovine serum (BSA) 10ng/mL (Sigma-Aldrich®) or Retinoic Acid 10ng/mL (Sigma-Aldrich®) in a humidified incubator at 37°C, 5% CO2 (Hera Cell 150i, Thermo Scientific).

4. EXPLANT ORGAN CULTURE

Agarose beads (Affi-gel Blue Gel, from Bio-Rad®) were impregnated with solutions containing bovine serum Albumin 200ng/µL (Sigma-Aldrich®); Recombinant Human Artemin 200ng/µL (Peprotech®) with GDNF family receptor alpha-3 (GFRα3) 200 ng/µL (R&D Systems®), in a dilution of 1:1; and Retinoic Acid 200ng/µL (Sigma-Aldrich®).

A minute amount of beads were placed on the mesenterium of the micro-dissected guts (E15.5). Supplemented RPMI medium was added to the samples and changed at 48h. Samples were incubated in a humidified incubator at 37°C, 5% CO2 (Hera Cell 150i, Thermo Scientific). Every 24h pictures were taken using a wide-field stereo fluorescent microscope [57]. Pictures were analysed with digital image processing software for microscope, AxioVision 4.8 [57].

Retinoic Acid Receptors β inhibitors were LE135 (Tocris-bioscience) and LE540 (Wako Chemicals) (10µM each) [51]. Blocking antibodies used were Goat anti-Mouse CXCL13/BLC/BCA-1

(R&D Systems®) and Mouse IgG anti-Human/Mouse CXCL12/SDF-1 (R&D Systems®) (1µg/mL each).

5. CONFOCAL MICROSCOPY

At 72 to 96H of explants organ cultures, samples were fixed with 4% Paraformaldehyde (Sigma-Aldrich®) for 15 minutes at room temperature. After fixed samples were washed with PBS-Triton 0.15% (Phosphate-Buffered Saline 7.4 from GIBCO® and Triton X-100 from Sigma-Aldrich®), blocked with PBS-Triton (0.15%) and incubated with 3% Goat, Rat or Rabbit serum (Abcam).

The following antibodies were used for immunofluorescence: Anti-GFP Rabbit IgG Fraction - Alexa Fluor 488 (Invitrogen™), Purified Rat anti-Mouse CD106 (429) (Biolegend®), Rat anti-Mouse CD4 - Alexa Fluor 647 (YTS 191.1) (AbD Serotec), Armenian Hamster anti-Mouse CD11c – Alexa Fluor 488 (N418) (Biolegend®), Neuronal class III β-tubulin (TUJ1) purified Rabbit (MRB-435P) (Covance) and Goat anti-Rat – Alexa Fluor 647 (Invitrogen™).

After the antibody incubations gradual dehydrations were done in consecutive concentrations of methanol (MeOH from Sigma-Aldrich®) in PBS-Triton, from 25, 50, 75 and 100%. After this process, specimens were incubated with a mix of BABB (Benzyl Alcohol:Benzyl Benzoate from Sigma-Aldrich®, in a 1:2 dilution) with MeOH in a 1:1 dilution. Next samples were incubated with 100% BABB.

Samples were mounted into a metal slide, with a hole drilled all the way through, BABB fill the hole and coverslips (Agar Scientific, No. 1.5) are attached using nail varnish [57].

For RALDH1 staining Samples were treated in 0.5% skim milk, 0.25% FBS and 0.5% Triton X-100 over 30 minutes at room temperature, and then washed with PBS 0.2% Tween. After the antibody incubations samples were washed in PBS and then distilled water. Samples were mounted into a metal slide, with a hole drilled all the way through, Mowiol fill the hole and coverslips (Agar Scientific, No. 1.5) are attached using nail varnish.

Images of whole-mount immunostained samples were acquired using a confocal microscope, Zeiss LSM 710, with objectives of 10x and 20x magnification (Objective EC "Plan-Neofluar" 10x/0.30 M27 and Objective "Plan-Apochromat" 20x/0.8 M27, respectively). Images acquired were analysed using Zeiss LSM Image Browser 4.2 as software.

6. GENE EXPRESSION ANALYSIS

Total RNA was extracted from sorted cells using RNAeasy Mini Kit (Qiagen), according to manufacturer's instructions. RNA was stored at -80°C.

Veriti 96-Well Thermal Cycler (Applied Biosystems™) was used for cDNA synthesis and PCR amplifications. cDNA synthesis was previously described [58]. Briefly, RNA was specifically retro-transcribed for 1h at 37°C by adding a mix containing 0.13µM specific reverse primer (Annex II), 50 mM KCl and 10 mM Tris-HCl at pH 8.3 (Applied Biosystems), 3.3 mM $MgCl_2$ (Applied Biosystems), 1 mM dNTPs (Applied Biosystems), 40 Units of RNase Block (Stratagene) and 35 Units of MuLV Reverse Transcriptase (Applied Biosystems) in a 15µL reaction. The reaction was stopped by a period of 3' incubation at 95°C.

6.1. First PCR Amplification

15µL of cDNA resulting from reverse transcription were amplified by an initial step of denaturation at 95°C for 10', followed by 15 cycles of amplification (45'' at 95°C, 1' at 60°C and 1'30'' at 37°C) with 50 mM KCl and 10 mM Tris-HCl at pH 8.3 (Applied Biosystems), 2 mM $MgCl_2$ (Applied Biosystems), 0.8 mM dNTPs (Applied Biosystems), 3 Units of AmpliTaq Gold DNA Polymerase (Applied Biosystems) and 0.015 µM specific primers (Annex II).

6.2. Real-Time Quantitative PCR

Real-time quantitative PCR was performed by adding 10 µL of 2x SYBR Green PCR Master Mix (Applied Biosystems) containing 4 µL of template and 6 µL of a primer mix with 0.25 µM of each specific primer (Annex II) in a 20 µL reaction volume using Rotor Gene 6000 (Corbett Life Science). After a denaturation step at 95°C for 10', the cycle profile used was 30'' at 95°C, 30'' at 60°C, and 45'' at 72°C for 50 cycles of amplification.

Threshold cycle (CT) was determined on the linear phase of the PCR curves, using the Corbett Rotor Gene 6000 Series Software (version 1.7). PCR products were run on a 1.5% agarose Gel Red (Biotium) gel.

7. GENOTYPING PCR

In order to genotype *Ret*$^{-/-}$ embryos, individual embryos were processed and their tail cut. Tail DNA was extracted using isopropanol/ethanol. Briefly, tail was incubated in Tail Lysis Buffer (Annex I) with 0.4mg/mL Proteinase K (Promega) at 56°C until the tissue was digested. Isopropanol (Sigma) was added and the samples were centrifuged for 20' at 16200g at 4°C. Supernatant was carefully removed, and the pellet washed with 70% ethanol (Merck), followed by a 10 min centrifugation at 4°C and 16200g. Finally, 70% ethanol was removed and the pellet let to air dry. DNA was then ressuspended in Milli Q water. Extracted DNA was amplified on a Veriti 96-Well Thermal Cycler (Applied Biosystems).

PCR consisted of a initial step of denaturation at 95°C for 10 min, followed by 35 cycles (3 sec at 96°C, 3 sec at 62°C and 5 sec at 68°C) with AmpliTaq Gold® Fast PCR Master Mix 1x (Applied Biosystems) and 0.25µM of each specific primer (Annex III) in a 20µL volume.

DNA extraction from adult tail was performed as described for embryo tails. Extracted DNA was amplified according to the following protocol: initial step of denaturation at 95°C for 10 min, followed by 35 cycles (30 sec at 94°C, 45 sec at 60°C and 1 min at 72°C) with 50mM KCl and 10mM Tris-HCl pH 8.3 (Applied Biosystems), 2.0mM $MgCl_2$ and 1.0mM dNTPs (Applied Biosystems), 0.5 units of AmpliTaq Gold DNA Polymerase (Applied Biosystems) and 0.25µM specific primers (Annex III) in a 20µL volume. PCR products were resolved on a 1.5% agarose Gel Red (Biotium) gel.

8. X-GAL STAINING OF EMBRYONIC TISSUES

In order to obtain heterozygous embryos, males for RARE-LacZ [59] were crossed with CD1 wild-type females. Pregnant females, at the chosen gestational day, were sacrificed and dissected. Embryos were then collected. Embryonic guts were microdissected in PBS and then fixed with 4% Paraformaldehyde (Sigma-Aldrich®) for 1 hour, at 4°C in the dark. Samples were then washed with rinse buffer, 3 times for 15 minutes. Rinse buffer: 5mM EGTA 2.0ml, 0.01% Deoxycholate 2.0 ml, 0.02% NP40 0.4 ml, 2mM $MgCl_2$ 0.4 ml, in a total volume of 200ml with PBS. After washing, tissues were incubated, in the dark, with the staining buffer at 37°C 3 to 7 hours or overnight. Staining buffer: 5mM $K_3Fe(CN)_6$ 0.4 ml, 5mM $K_4Fe(CN)_6$ 0.4 ml, 5mM EGTA 0.4 ml, 0.01% Deoxycholate 0.4 ml, 0.02% NP40 80µl, 2mM $MgCl_2$ 80µl, in a total volume of 39 ml with PBS; add 1ml of stock X-gal solution to a final concentration of 1mg/ml. Reagents purchased from Sigma-Aldrich®.

9. TIME LAPSES

Embryonic GFP^{pos} guts (E15.5) were laid on a cell strainer [57], room temperature supplemented RPMI medium was added to the samples. Different molecules were added to the medium, such as bovine serum Albumin, Retinoic Acid or Retinoic Acid Receptors β antagonists, LE135 and LE540 (25ng each). Samples were incubated in a humidified incubator at 37°C, 5% CO_2 (Hera Cell 150i, Thermo Scientific) for 24h. After this period, time-lapse analysis was performed taking advantage of a Temperature control box 37–2 digital, Heating Insert P and Incubator P from PECON and a wide-field stereo fluorescent microscope (Zeiss Stereo Lumar.V12 with a Zeiss Neolumar S 0.8x objective). Time-lapse analyses were performed at

37°C during 1h; pictures were taken every minute using the digital image processing software AxioVision 4.8 (Zeiss). All the results acquired were analysed using ImageJ software.

10. MICROARRAYS

RNA was extracted from stimulated cells, with BSA and RA, that were previously sorted, $CD45^{pos}$ and $CD45^{neg}$ populations. RNA extraction was done using RNAeasy Mini Kit (Qiagen), according to manufacturer's instructions. RNA was stored at -80°C.

Microarrays were done at Instituto Gulbenkian para a Ciência (IGC) in Gene Expression unit using Scanner 3000 7G with Autoloader, Hybridization Oven 640 and Bioanalyzer 2100.

Analysis was done using Partek® Express™, Affymetrix™ Edition™ software.

11. STATISTICS

Statistical analysis was performed on Microsoft Office Excel 2007. Variance was calculated using F-test. Student's t-test was performed both on homoscedastic and on heteroscedastic populations with the appropriate correction. Significance was defined at $p < 0.05$ and error bars in graphs represent Standard Error of the Mean (SEM).

III. RESULTS

1. ANIMAL MODEL AND SLOs PRIMORDIA ANALYSIS

Despite recent progress on the understanding of lymphoid organogenesis, the difficulty of isolating SLO primordia and the lack of reductionist *in vitro* approaches has hindered advances in the field. We overcame previous limitations, focusing on the identification of mechanisms controlling lymphoid organogenesis in general and on the role of Retinoic Acid in particular.

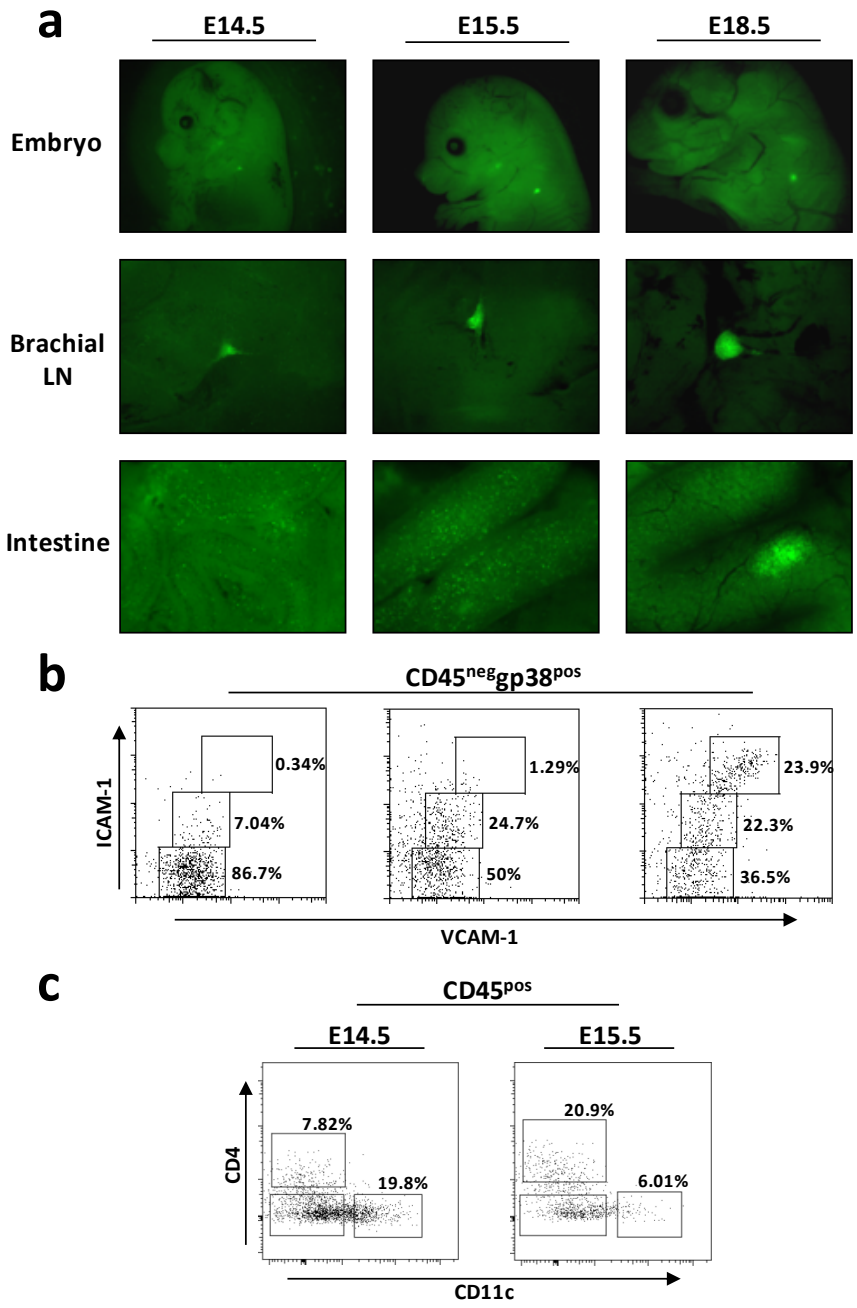


Figure 3. **Haematopoietic and stromal cell populations in anlagen lymph nodes.** hCD2-GFP mice were used to identify lymphoid structures by stereo fluorescence microscopy. **a.** hCD2-GFP embryos were analysed (top panels). Results show details of brachial lymph node (middle panels) and details of Peyer's patch formation (Bottom panel) **b.** Representative FACS plot analysis for stromal cell populations. **c.** Representative FACS plots analysis for haematopoietic cell populations at different days of embryonic life show $CD45^{pos}CD4^{pos}CD11c^{neg}$ Lymphoid Tissue inducer (LTi) and $CD45^{pos}CD4^{neg}CD11c^{pos}$ Lymphoid Tissue initiator (LTin) cells.

We used reporter transgenic hCD2-GFP mice[5] that allow visualisation, microdissection and analysis of developing foetal lymphoid structures (Figure. 3a, b, c). During PP formation the distribution of the GFP^{pos} cells is modified. Initially these cells are randomly distributed in the mid-gut, but later on they aggregate and form large clusters of cells that constitute the primordial of PP (Fig.3a). Cervical, axillary and brachial LNs were microdissected from E14.5, E15.5 and E18.5 hCD2-GFP embryos (figure 3a). In order to characterise the phenotype of Lymphoid Tissue organizer (LTo) cells throughout development we analysed the microdissected primordia through flow cytometry. Results revealed that the surface markers VCAM-1 and ICAM-1 are modulated: stromal cells are initially $VCAM\text{-}1^{neg}ICAM\text{-}1^{neg}$, afterwards they start acquiring these molecules, becoming intermediately positive, and at later stages a significant proportion exhibits a mature $VCAM\text{-}1^{hi}ICAM\text{-}1^{hi}$ phenotype (figure 3b). Besides the analysis of LTo cells, and based on the expression of the surface markers CD4 and CD11c, FACS analysis revealed two main haematopoietic populations present in the LN primordia that were previously described in the literature, LTi and LTin cells (figure 3c). The proportion of Lymphoid Tissue inducer (LTi) cells, $CD45^{pos}CD4^{pos}CD11c^{neg}$ cells, increased throughout development, while the percentage of Lymphoid Tissue initiator (LTin) cells, $CD45^{pos}CD4^{neg}$ $CD11c^{pos}$ cells, were higher in an early phase and diminished later, which is consistent with the idea that LTin cells may be primarily involved in early events of SLO development (figure 3b). Similarly to what was observed in the LN primordia, FACS analysis of embryonic gut revealed the same constitution of haematopoietic population, as well as the same evolution of the proportion of both of these throughout development (Annex IV, supplementary figure 1).

2. RETINOIC ACID AND ENTERIC LYMPHOID ORGAN FORMATION

Retinoic Acid metabolism is a complex process in which several enzymes are involved. The RALDH family of dehydrogenases is responsible for most RA production during early embryogenesis [44] while identification of a target responsive cell for RA is the recognition of RA responsive elements [42].

Confocal microscopy analysis of E15.5 intestine revealed that RALDH1 is highly expressed in this organ and that this expression is not confined to hCD2-GFP cells (figure 4a). The expression profile of this enzyme follows the pattern of a nervous network, which correlates with the crucial function of RA in the development of nervous cells [44] (annex V, supplementary figure 2). In addition, quantitative PCR of E15.5 gut cells, showed that RALDH2 is also expressed in embryonic intestines (Figure 4b). Finally, taking advantage of transgenic mice that express LacZ under RARE regulatory elements, RARE-LacZ [60], we found that throughout enteric

development the pattern of RA target cells is characterised by a seemingly random distribution (figure 4c).

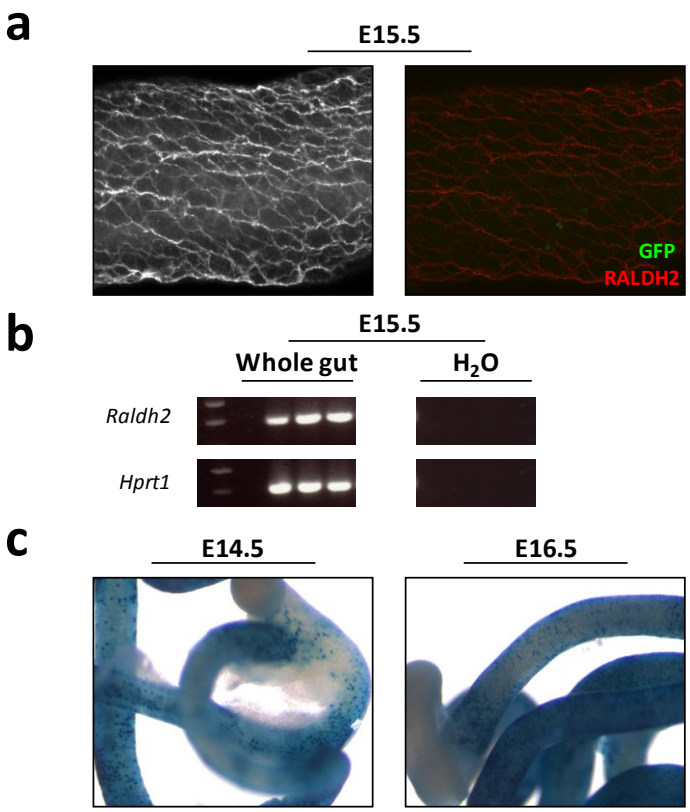


Figure 4. **Retinoic Acid (RA) expression in the embryonic gut.** Microscopy techniques and RARE-LacZ mice were used to identify cells that produce RA and cells that have RA function in the intestine. **a.** E15.5 embryonic guts analysed by confocal microscopy show that the cells that express RALDH1, enzyme involved in the RA synthesis, are not GFP ^{pos} haematopoietic cells, and that they form a network through the wall of the gut. **b.** qPCR for RALDH2, enzyme involved in the RA synthesis. **c**. RARE-LacZ mice were analysed showing that RA target cells are found in the gut throughout development.

The finding that RALDH1, RALDH2 and RA target cells are found in the embryonic gut led us to investigate whether RA could impact on enteric lymphoid organ formation. In order to address that, we took advantage of a reductionist explant organ culture system using embryonic hCD2-GFP transgenic intestines. Briefly, agarose beads were impregnated with either BSA or RA and were incubated with E15.5 hCD2-GFP guts. Strikingly, we found that RA impregnated beads induced the accumulation of haematopoietic GFP^{pos} cells (Figure 5a). Moreover, this process was efficiently inhibited by antagonists of Retinoic Acid Receptor β (figures 5a, b and c) demonstrating that in this experimental set-up haematopoietic cell clustering is specifically mediated by RA signalling.

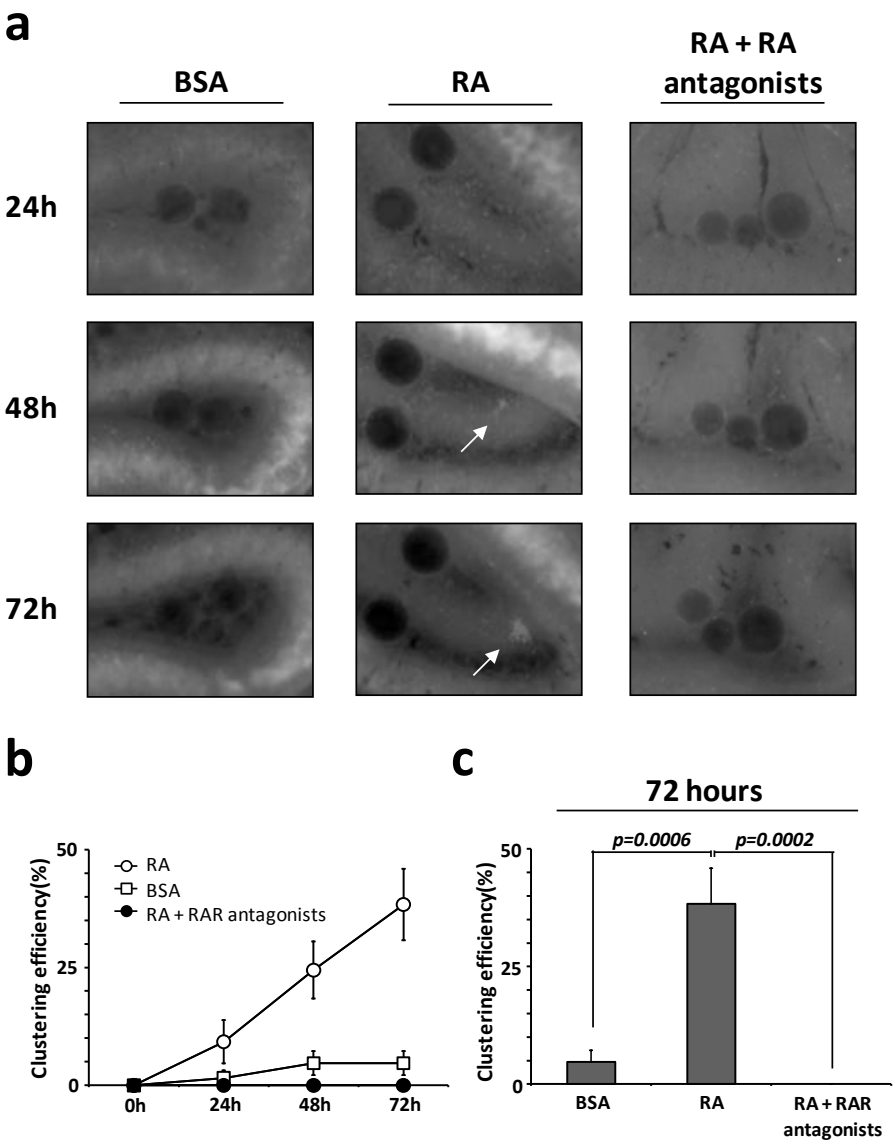


Figure 5. RA induces haematopoietic cell clusters in enteric explant organ cultures. Embryonic guts from E15.5 hCD2-GFP embryos were cultured with agarose beads impregnated with BSA, RA and RA plus antagonists of RA receptors β. Analysis was performed by stereo microscopy. **a.** Results show cluster formation over 72 hours of enteric explants organ culture. Arrows indicate GFP positive cell clusters. **b.** Kinetic analysis of haematopoietic cell clustering efficiency. **c.** Haematopoietic cell clustering efficiency at 72h. T-student p-values are indicated. Error bars indicate standard errors. BSA n=16; RA n=15; RA+RAR antagonists n=9.

The formation of haematopoietic GFP^{pos} cell clusters in response to RA led us to investigate their cellular composition in comparison to ectopic lymphoid structures that are efficiently induced by RET ligands [5]. Immunohistochemical staining of RA induced aggregates revealed that, similarly to RET ligands induced clusters, mesenchymal cells in their vicinity expressed high levels of VCAM1, a sign of mature LTo cells (figure 6a). Levels of expression of VCAM-1 in the two clusters were compared, showing no statistical significant difference between them (figure 6b). Remarkably, we found that cell aggregates, whether formed by ARTN+GFRα3 as

well as by RA, were composed of both LTi ($CD45^{pos}CD4^{pos}CD11c^{neg}$) and LTin ($CD45^{pos}CD4^{neg}CD11c^{pos}$) (figure 6c and d).

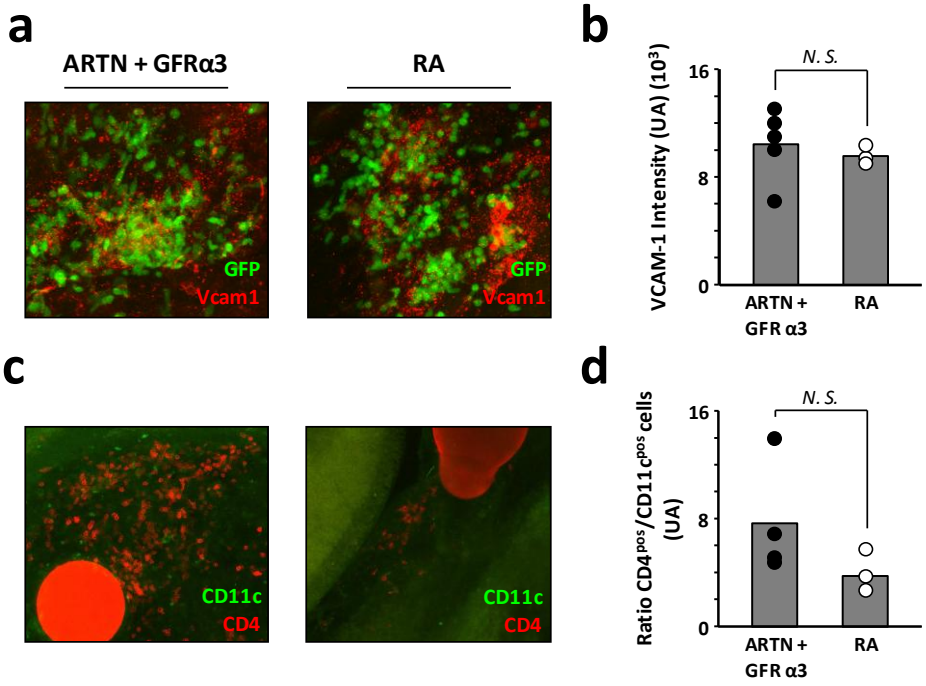


Figure 6. **Confocal microscopy analysis of ARTN and RA induced cell clusters.** Immunostaining of haematopoietic cell aggregates at 72h of enteric explant culture. **a.** VCAM-1 (Red) and GFP (Green) staining. Results show that, similarly to RET signalling, RA signalling induces stromal cell maturation. **b.** Comparision of VCAM-1 mean intensity of expression between ARTN and RA induced clusters. **c.** CD4 (Red) and CD11c (Green) staining. Results indicate that even though the amount of LTin and LTin cells is different in ARTN and RA induced clusters, the ratio of these two populations is similar in both types of clusters.

3. RELATIONSHIP BETWEEN RA AND RET SIGNALLING

The signalling axis RET/ARTN was described to be important for PP development [5], and it was suggested that this signalling is likely to be the triggering event leading to PP primordium formation [30]. Our results show that, similarly to RET ligands, RA induces enteric haematopoietic cell clustering and consequent stromal cell maturation. Thus, we investigated whether RET and RA signalling axes act independently of each other.

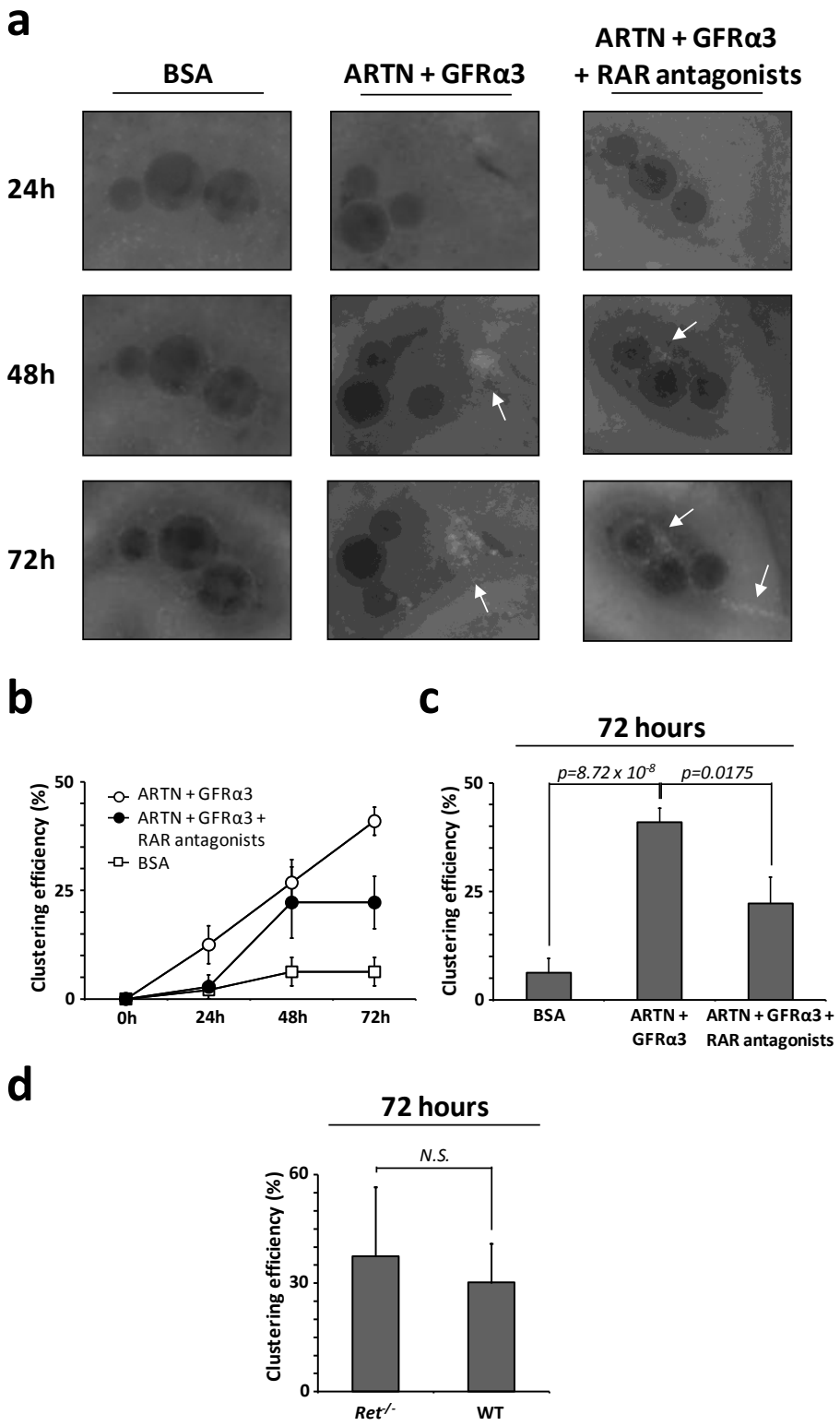


Figure 7. **ARTN induced haematopoietic cell clustering is affected by blockage of RA signalling**. Guts from E15.5 hCD2-GFP embryos were cultured with agarose beads impregnated with BSA, ARTN+GFRα3 or ARTN+GFRα3 plus RARβ antagonists. **a.** Representative images of cluster formation. Arrows indicate GFP cell clusters. **b.** Kinetics of clustering efficiency. **c.** Clustering efficiencies were analysed at 72h of explant gut culture.. T-student p values are indicated. Error bars indicate standard errors. BSA n=12; ARTN+GFRα3 n=14; ARTN+GFRα3+RAR antagonists[7] n=9. **d.** Clustering efficiencies for E14.5 *Ret*$^{-/-}$ and WT embryos were analysed at 72h of explant organ culture. T-student p values are indicated. Error bars indicate standard errors. *Ret*$^{-/-}$ n=3; WT n=4.

In order to achieve that, E15.5 hCD2-GFP guts were cultured with agarose beads impregnated with ARTN+GFRα3 and RARβ antagonists were added to the culture medium (figure 7a, b, c). Interestingly, we found that RA signalling block impairs cluster formation by RET ligands (figure 7a, b, c). In addition, explant organ culture of intestines from *Ret*$^{-/-}$ and WT embryos with RA impregnated beads.Show that in *Ret*$^{-/-}$ and WT intestines RA yields similar clustering efficiency (figure 7 d). Thus, these data suggest that RA acts independently of RET signalling; but that RA cues are still required for full activity of RET ligands.

4. RA SINALS DO NOT MEDIATE ENTERIC HAEMATOPOIETIC MOTILITY

Taking into consideration that colonising enteric haematopietic cells are highly motile [5] and that RA is required for the proper migratory behaviour of enteric neural crest derived cells during embryogenesis [61], we investigated whether RA is also involved in haematopoietic cell motility in the gut. In order to address that, we took advantage of hCD2-GFP transgenic model and performed time-lapse analysis. Briefly, embryonic guts were incubated with BSA or RA during 24h and then images were taken every 60 seconds during 1h. We found that increased RA concentrations did not change the speed (6 µm min^{-1}) or the motility patterns of enteric haematopoietic cells (figure 8a, b; Supplementary video 1 and 2, annex VI). These results suggest that RA signaling, is not involved in haematopoietic cell motility in the intestine.

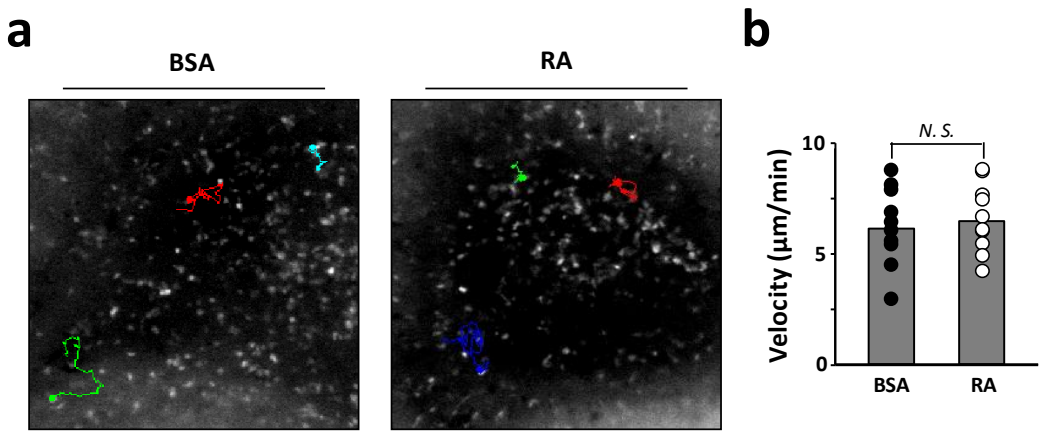


Figure 8. Effect of RA stimulation in the motility of the enteric haematopoietic cells. Guts from E15.5 hCD2-GFP embryos were stimulated with BSA or RA during 24h and subsequently time-lapses stereo analysis was performed. **a.** Details of time-lapse analysis showing examples of three cell tracks over a 1 hour period of. **b.** Results show mean cell velocities. T-student testes were done. BSA n=10; RA n=10.

5. ROLE OF CXCL12 AND CXCL13 IN RA INDUCE CLUSTERING

Previous studies have indicated that chemokines such as CXCL13 are important for SLO development, since *Cxcr5*$^{-/-}$ [62] and *Cxcl13*$^{-/-}$ [63] embryos lack all LNs. Moreover, it was recently shown that RA activation of stromal cells could lead to CXCL13 expression by the latter [51]. Thus, we investigated whether the chemokines CXCL13 and CXCL12 (that we have found to be expressed in the gut (unpublished data)), could mediate RA signalling during lymphoid structure formation. In order to address this question we used enteric explant organ cultures with RA impregnated beads and CXCL12 and CXCL13 blocking antibodies were added to the cultures (figure 9a, b and c).

Surprisingly, we found that blocking these chemokines had no impact on the cluster efficiency of RA (figure 9b and c). In addition, we performed quantitative RT-PCRs from E15.5 gut cells stimulated over a 24h period with either BSA or RA. We found that expression of CXCL12 and CXCL13 was not increased with RA treatment; on the contrary, CXCX13 was down-regulated upon RA stimulation (figure 9d). Altogether these results show that, contrary to LN development, CXCL13, and CXCL12, do not seem to mediate RA signalling during enteric structure formation in embryogenesis.

6. MICROARRAY ANALYSIS OF RA STIMULATED GUT POPULATIONS

Since the chemokine CXCL13, a down-stream molecule of RA signalling in LN development, was not modulated by RA in the intestine, we investigated which other molecules could play this role in the gut.

In order to address this we purified gut populations by flow cytometry, sorting CD45^{pos} and CD45^{neg} cells. Consecutively, these populations were stimulated with BSA or RA for 6 hours. For this analysis an unadjusted p-value < 0.05 and a fold change of 1.5 were considered. Surprisingly, we found that despite RA activity in CD45^{neg} cells, as revealed by RAR upregulation upon treatment, chemokine levels were not significantly modified by RA stimulation. Conversely, the subcategories which were more modulated by the RA stimulus, in the biological process, were developmental process and growth. In the cellular component the subcategory which expression was more affect was extracellular region, followed by the extracellular part region. Remarkably, in the molecular function category the subcategories more modulated by the RA stimulus were transcription regulatory activity, followed by binding. Strikingly, one of the genes which expression was more upregulated upon RA stimulus was Rarβ, Retinoic Acid Receptor beta. This result confirms the conclusion reached before, that RA promotes its own synthesis through a positive feedback (figure 10).

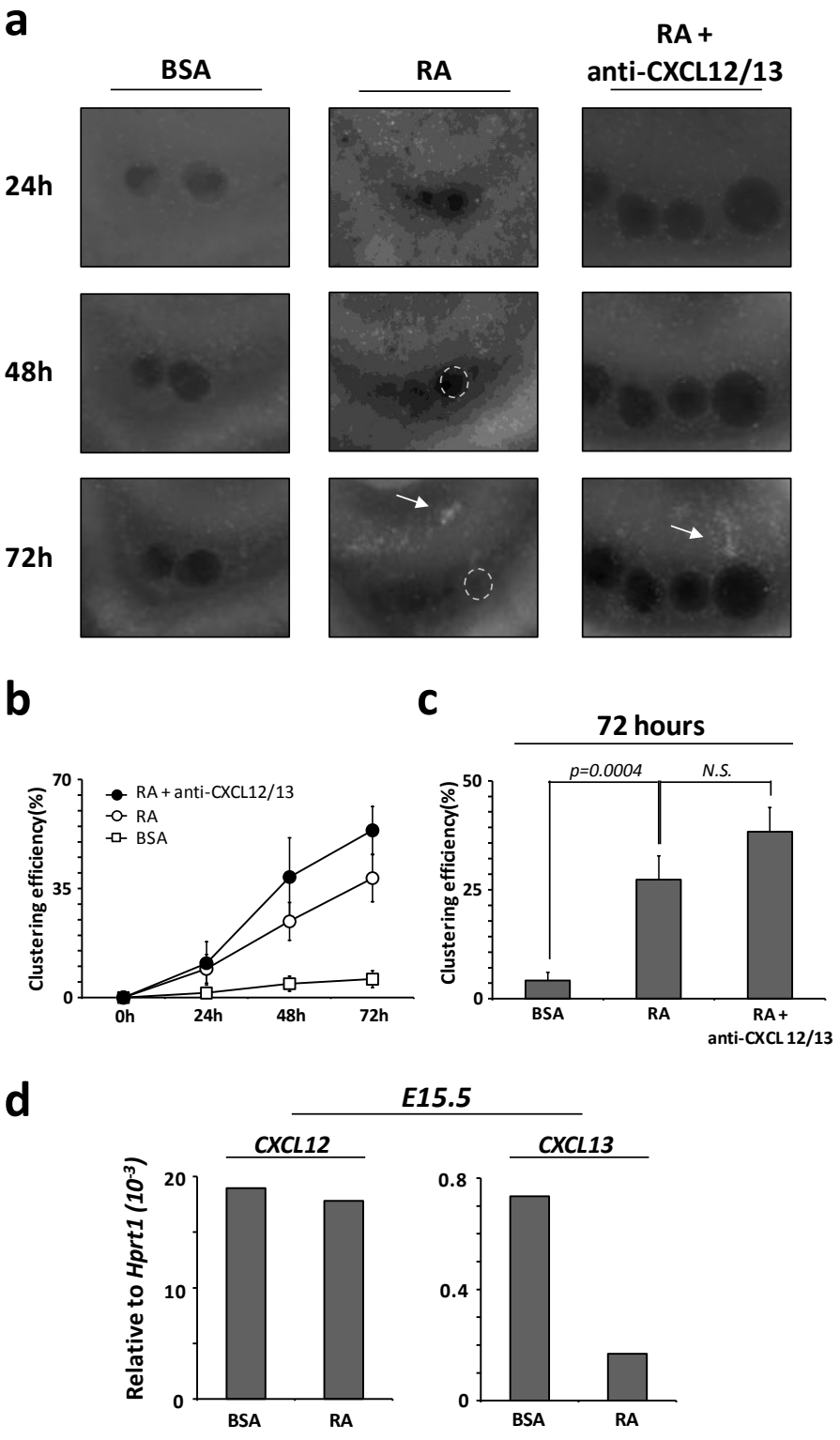


Figure 9. Influence of CXCL12 and CXCL1313 in RA induced clustering . Guts from E15.5 hCD2-GFP embryos were cultured with agarose beads impregnated with BSA, RA or RA plus CXCL12 and CXCL13 blocking antibodies. **a.** Representative experiment of cluster formation. Arrows indicate GFP cell clusters. **b.** Kinetics of the clustering efficiency. **c.** Clustering efficiencies were analysed at 72h. T-student p values are indicated. Error bars indicate standard errors. BSA n=17; RA n=15; RA+anti-CXCL12+anti-CXCL13n=6. **d.** CXCL12 and 13 levels in RA stimulated gut samples. qPCRs of whole gut cell suspensions treated with BSA or RA for 24h.. qPCRs for the chemokinesCXCL12 and 13 show no upregulation of these genes in samples stimulated with RA.

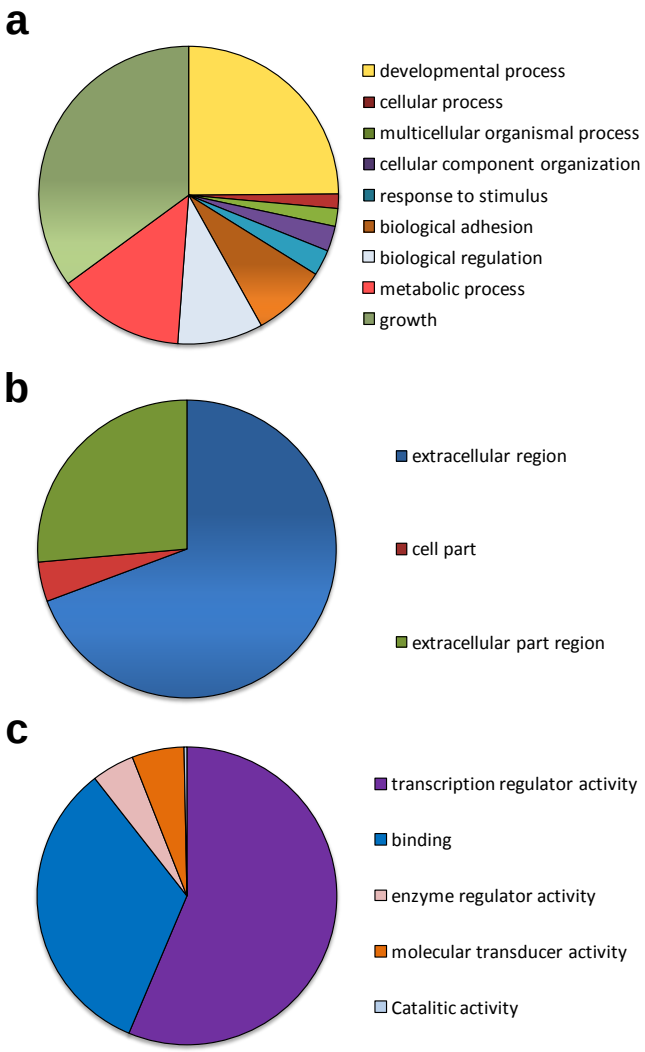


Figure 10. Microarray analysis of CD45^{neg} gut cells stimulated with RA. E15.5 gut cells were sorted as CD45^{pos} and CD45^{neg} populations and then each population was treated with BSA or RA for 6h. Microarray analysis of the two populations was performed with an unadjusted p-value < 0.05 and fold change of 1.5. **a.** Piechart representation of the differently expressed genes, within the *biological process* category. **b.** Piechart representation of the differently expressed genes, within the *cellular component* category. **c.** Piechart representation of the differently expressed genes, within the *molecular function* category.

Interestingly, we found that CD45^{pos} haematopoietic cells were the cell type that exhibited the larger list of modulated genes by RA stimulation. The subcategories which were more modulated by the RA stimulus, in the biological process, were death and response to stimulus. However, in the cellular component the subcategories which expression was more affect were extracellular region and cell part. Strikingly, in the molecular function category the subcategory more modulated by the RA stimulus was chemorepellent activity, followed by antioxidant activity and binding (figure 11).

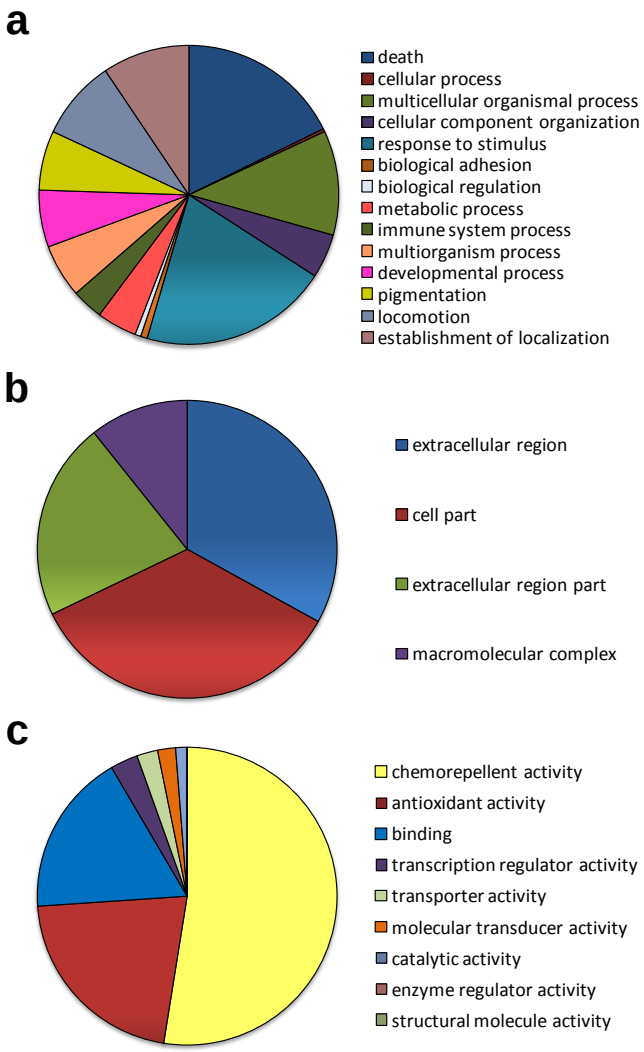


Figure 11. Microarray analysis of CD45^{pos} gut cells stimulated with RA. E15.5 gut cells were sorted as CD45^{pos} and CD45^{neg} populations and then each population was treated with BSA or RA for 6h. Microarray analysis of the two populations was performed with an unadjusted p-value < 0.05 and fold change of 1.5. **a.** Piechart representation of the differently expressed genes, within the *biological process* category. **b.** Piechart representation of the differently expressed genes, within the *cellular component* category. **c.** Piechart representation of the differently expressed genes, within the *molecular function* category.

Noticeably, in the CD45^{pos} subset, Ly6a, lymphocyte antigen 6 complex, locus A (also known as Sca-1) was highly down-modulated upon RA treatment (figure 12b).

Sca-1, together with other makers such as c-kit, is a marker commonly used to isolate pure hematopoietic stem cells (HSCs) population. As HSCs commit to lymphoid progenitors, Sca-1 expression decreases [64]. Studies conclusively demonstrated that Sca-1, in addition to being a marker of HSCs, regulates the developmental program of HSCs and specific progenitor populations [64]. Taking into consideration that RA stimulation in the CD45^{pos} subset results in a downregulation of Sca-1, we suggest that RA signalling might be involved in enteric

haematopoietic cell differentiation. In order to understand whether Sca-1 correlates with differentiation of enteric haematopoietic cells, we analysed its expression profile in E15.5 intestines. Remarkably, preliminary experiments revealed that while Sca-1 is expressed in LTin cells and in $CD3^{neg}CD4^{neg}CD11c^{neg}cKit^{pos}IL7R\alpha^{pos}\alpha4\beta7^{pos}$ (double negative (DN)) cells, $CD45^{pos}CD4^{neg}CD11c^{neg}$ cells, LTi cells do not express this molecule (figure 12c). Altogether our results show that RA signalling leads to a genetic signature that differs from what was previously described during LN development [51]

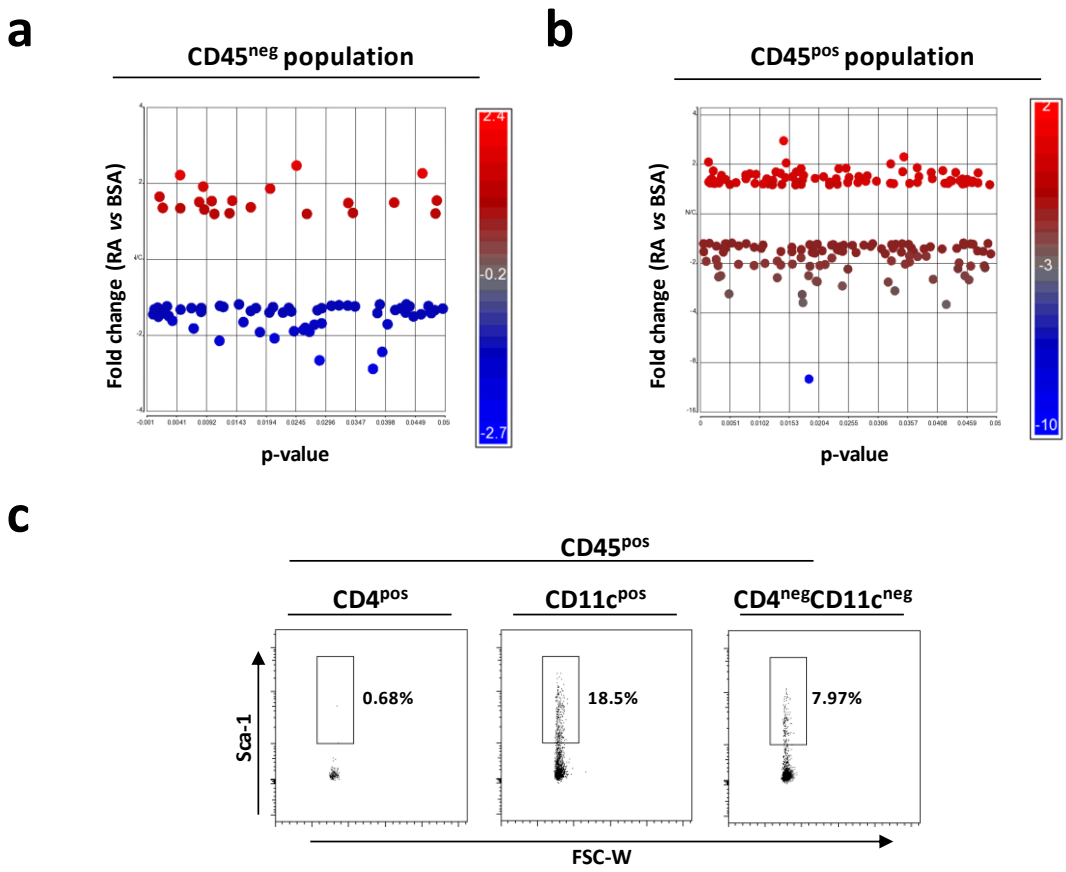


Figure 12. Microarray analysis of $CD45^{neg}$ and $CD45^{pos}$ gut cells stimulated with RA and Sca-1 expression profile within haematopoietic populations. E15.5 gut cells were sorted as $CD45^{pos}$ and $CD45^{neg}$ populations and each population was treated with BSA or RA for 6h. Microarray analysis was performed with an unadjusted p-value < 0.05 and fold change of 1.5. **a.** Mathematical representation of fold change *versus* p-value showing the genes expressed in the $CD45^{neg}$ subset upon RA stimulation. **b.** Mathematical representation of fold change *versus* p-value showing the genes expressed in the $CD45^{pos}$ subset upon RA stimulation. Sca-1 has been revealed as the most differentially expressed gene. **c.** Sca-1 expression profile within haematopoietic population. Representative FACS plots for Sca-1 expression. Left panel LTi cells; middle panel: LTin cells; right panel: $CD45^{pos}CD4^{neg}CD11c^{neg}$ double negative (DN) population.

IV. DISCUSSION

Development of enteric SLOs depends on cellular and molecular cues that coordinate clustering of different cell types. Similarly to lymph node development, the cellular and molecular mechanisms implicated in PP development are well characterized, relying on interactions between cells from haematopoietic and mesenchymal origin. Despite the fact that these processes are not entirely identical and even require different players, there are obvious parallels between LN and PP genesis.

RET signalling is one of the pathways involved in the onset of PP development. In late 2009 RA signalling was also revealed to be involved in the initial phases of LN genesis[5, 51], however, its role in enteric lymphoid organogenesis remains elusive.

Herein, we show that RA signalling modulates the behaviour of haematopoietic cells involved in PP development. Moreover, we found that conversely to LN development, RA cues are not involved in the induction of chemokine expression on stromal cells. Instead, we found that RA signalling impinges a genetic signature on haematopoietic cells that correlates with cell differentiation.

We found RALDH1 and 2 expressions in the embryonic intestine. Surprisingly, the pattern of enteric RA production (revealed by RALDH1 expression) and activity (revealed by RARE expression) do not entirely overlap. RADH1 expression is restricted to enteric nervous cells, while GFP haematopoietic cells do not express this Dehydrogenase. These findings are in line with the idea that nervous cells in the vicinity of developing LNs are the main source of RA production [51]. However, *Gdnf* and *Gfra1* null embryos that fail to develop a myenteric nervous plexus have normal PP development arguing against a possible role of nervous cell origin RA in PP development [5, 65, 66]. However, parasympathetic and/or sympathetic nervous axons, still present in the guts of these mutants, may provide such retinoic acid cues for PP formation. Future RALDH immunostainings in these mutants will shed light on what other cell types may produce RA and will allow us to selective ablate RALDH activity on those cells by using a conditional knock out strategy [67].

Interestingly, we found that the pattern of RARE positive cells was reminiscent of the distribution of GFP haematopoietic cells in the developing gut. This finding, together with the RA induced modulation of the genetic signature of haematopoietic cells, strongly suggests that haematopoietic cells are likely to be a direct target of RA signalling. In the near future, we plan to assess this hypothesis by flow citometry taking advantage of RARE-LacZ transgenic guts [60]. In this context, the identification of RA target haematopoietic cell subsets is paramount for the understanding of RA activity in the gut $CD45^{pos}$ cells.

Explant organ culture of E15.5 intestines showed that RA signalling induces the aggregation of a full complement of haematopoietic subsets that in turn activate resident stromal cells. In

agreement with this observation, blockage of this signalling axis by RARβ antagonists result in the abolishment of haematopoietic cell clustering induced by RA.

The formation of haematopoietic GFP^{pos} cell clusters in response to RA and the fact that ectopic lymphoid structures can be efficiently induced by RET ligands [5], led us to investigate whether these signalling axes act independently of each other. We found that in the absence of RET signalling, RA can efficiently induce the formation of ectopic lymphoid structures. Moreover, blockage of RA signalling impaired to some extent RET ligands' induced clustering Thus, we would like to suggest that that RA acts independently of RET signalling, but that to some degree, RA cues are still required for full activity of RET ligands.

Bearing in mind that RET and RA signalling axes were shown to be required for the proper migratory behaviour of enteric neural-crest derived cells [61], we investigated whether RA could modulate haematopoietic cell motility. We found that cells stimulated either with BSA or RA exhibit a remarkable similar random motility averaging identical speeds (6 μm min^{-1}). Thus, conversely to nervous cells, RA is not involved in the motility pattern of enteric hematopoietic cells. These results are in line with mounting evidence suggesting that molecular mechanisms historically ascribed to specific tissues can be more widely, to differentially orchestrate the function of systems derived from different germ layers [68].

Previous studies have indicated that RA activation of stromal cells leads to CXCL13 expression [51], a chemokine that is important for SLO development [51, 63] . Surprisingly, we found that blocking these chemokines had no impact on RA clustering efficiency. Additionally, the expression of the chemokines CXCL12 and CXCL13 was not increased upon RA stimulation. Altogether, these results demonstrate that despite its capacity of modulating haematopoietic cell behaviour, RA signalling cues are not orchestrated similarly in LN and PP genesis.

Consequently, we investigated which other molecules could be down stream of RA signalling in the gut. Microarrays analysis was performed in CD45neg and CD45pos enteric populations upon RA treatment. Both populations responded to the treatment as revealed by up-regulation of RARβ, nuclear receptor for RA [60]. However, despite RA activity, no significant modification of chemokine levels was observed in $CD45^{neg}$ cells, supporting the idea that RA impinges a differential genetic signature during LN and PP genesis. Interestingly, we found that RA stimulation of $CD45^{pos}$ haematopoietic cells induced a large array of modulated genes. Among those, Ly6a (commonly known by Sca-1) was highly down modulated. Sca-1 is usually used as a hematopoietic stem cells (HSCs) marker, and as HSCs commit to lymphoid progenitors, Sca-1 expression decreases [64]. Interestingly we found that that LTin and $CD3^{neg}CD4^{neg}CD11c^{neg}cKit^{pos}IL7R\alpha^{pos}\alpha4\beta7^{pos}$ (double negative (DN)) cells, were Sca-1pos; while LTi cells do not express this molecule. These preliminary results raise the hypothesis that

rather than being involved in the motility of haematopoietic cells or chemokine induction in the gut, RA might modulate the differentiation of $CD3^{neg}CD4^{neg}CD11c^{neg}cKit^{pos}IL7R\alpha^{pos}\alpha4\beta7^{pos}$ progenitor cells. We plan to experimentally address this question by studying the outcome of different haematopoietic subsets in three-dimension cultures with bona-fide mesenchymal LTo cells with increased or reduced RA signalling by adding respectively, RA or RARβ antagonists to the cultures. This approach will elucidate whether RA promotes the differentiation of rather undifferentiated haematopoietic progenitors. In order to achieve these goals we will use human CD2-GFP and/or human CD2-DsRed transgenic mice that express respectively, GFP and DsRed in all haematopoietic cell subsets aggregating at sites of SLO development. Using an educated reductionist approach, *ex vivo* haematopoietic cell subsets will be co-cultured with bona-fide anlagen LN stromal cells and differentiation evaluated by Fluorescence Activated Cell Sorting (FACS) analysis.

Our data reveal that the RA axis acts independently of RET signalling, but that RA cues are still required for full efficiency of RET ligands. In addition, our results suggest that RA might modulate the differentiation of enteric haematopoietic cells. Taken together, we would like to propose a model whereby RA signals would induce differentiation of $CD3^{neg}CD4^{neg}CD11c^{neg}cKit^{pos}IL7R\alpha^{pos}\alpha4\beta7^{pos}$ progenitor cells into LTi cells. In physiological concentrations, RA would be a limiting factor and therefore RET signalling on LTin cells would still be required for PP triggering. In this context, RARβ block would result in impairment of *de novo* formation of LTi cells, and would therefore impact on RET ligands clustering activity. Conversely, localised increased concentrations of RA would promote focal LTi development and consequent stromal cell maturation.

The findings in this thesis provide evidence that RA signalling modulates the activity of haematopoietic cells that participate in PP organogenesis. Thus, RA emerges as a novel molecule in the development of these lymphoid structures and opens new exciting research avenues on the integration of this signalling axis with other seemingly non related pathways.

REFERENCES

1. Fu, Y.X. & Chaplin, D.D. Development and maturation of secondary lymphoid tissues. *Annu Rev Immunol* **17**, 399-433 (1999).
2. Paul, W.E. Fundamental Immunology (ed. Wilkins, L.W.) (Philadelphia, 2003).
3. Taylor, R.T. & Williams, I.R. Lymphoid organogenesis in the intestine. *Immunol Res* **33**, 167-81 (2005).
4. Randall, T.D., Carragher, D.M. & Rangel-Moreno, J. Development of secondary lymphoid organs. *Annu Rev Immunol* **26**, 627-50 (2008).
5. Veiga-Fernandes, H. et al. Tyrosine kinase receptor RET is a key regulator of Peyer's patch organogenesis. *Nature* **446**, 547-51 (2007).
6. Friedberg, S.H. & Weissman, I.L. Lymphoid tissue architecture. II. Ontogeny of peripheral T and B cells in mice: evidence against Peyer's patches as the site of generation of B cells. *J Immunol* **113**, 1477-92 (1974).
7. Cornes, J.S. Number, size, and distribution of Peyer's patches in the human small intestine: Part I The development of Peyer's patches. *Gut* **6**, 225-9 (1965).
8. Ohtani, O. & Ohtani, Y. Organization and developmental aspects of lymphatic vessels. *Arch Histol Cytol* **71**, 1-22 (2008).
9. Muramatsu, M. et al. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* **102**, 553-63 (2000).
10. Kinoshita, K., Harigai, M., Fagarasan, S., Muramatsu, M. & Honjo, T. A hallmark of active class switch recombination: transcripts directed by I promoters on looped-out circular DNAs. *Proc Natl Acad Sci U S A* **98**, 12620-3 (2001).
11. Bergqvist, P., Gardby, E., Stensson, A., Bemark, M. & Lycke, N.Y. Gut IgA class switch recombination in the absence of CD40 does not occur in the lamina propria and is independent of germinal centers. *J Immunol* **177**, 7772-83 (2006).
12. Mebius, R.E. Organogenesis of lymphoid tissues. *Nat Rev Immunol* **3**, 292-303 (2003).
13. Janet Rossant, P.P.L.T. Mouse Deveolpment - Patterning, Morphogenesis and Organogenesis (ed. Press, A.) (Canada, 2002).
14. Vondenhoff, M.F., Kraal, G. & Mebius, R.E. Lymphoid organogenesis in brief. *Eur J Immunol* **37 Suppl 1**, S46-52 (2007).
15. Blum, K.S. & Pabst, R. Keystones in lymph node development. *J Anat* **209**, 585-95 (2006).
16. Cupedo, T. et al. Presumptive lymph node organizers are differentially represented in developing mesenteric and peripheral nodes. *J Immunol* **173**, 2968-75 (2004).
17. Mebius, R.E., Rennert, P. & Weissman, I.L. Developing lymph nodes collect CD4+CD3- LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. *Immunity* **7**, 493-504 (1997).
18. Adachi, S., Yoshida, H., Kataoka, H. & Nishikawa, S. Three distinctive steps in Peyer's patch formation of murine embryo. *Int Immunol* **9**, 507-14 (1997).
19. Cupedo, T., Kraal, G. & Mebius, R.E. The role of CD45+CD4+CD3- cells in lymphoid organ development. *Immunol Rev* **189**, 41-50 (2002).
20. Cupedo, T. & Mebius, R.E. Cellular interactions in lymph node development. *J Immunol* **174**, 21-5 (2005).
21. Kim, D. et al. Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. *J Exp Med* **192**, 1467-78 (2000).
22. Adachi, S. et al. Essential role of IL-7 receptor alpha in the formation of Peyer's patch anlage. *Int Immunol* **10**, 1-6 (1998).
23. Benezech, C. et al. Ontogeny of stromal organizer cells during lymph node development. *J Immunol* **184**, 4521-30.

24. Yoshida, H. et al. Different cytokines induce surface lymphotoxin-alphabeta on IL-7 receptor-alpha cells that differentially engender lymph nodes and Peyer's patches. *Immunity* **17**, 823-33 (2002).
25. Honda, K. et al. Molecular basis for hematopoietic/mesenchymal interaction during initiation of Peyer's patch organogenesis. *J Exp Med* **193**, 621-30 (2001).
26. Luther, S.A., Ansel, K.M. & Cyster, J.G. Overlapping roles of CXCL13, interleukin 7 receptor alpha, and CCR7 ligands in lymph node development. *J Exp Med* **197**, 1191-8 (2003).
27. Yoshida, H. et al. Expression of alpha(4)beta(7) integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lymphotoxin producer, NK, and dendritic cells. *J Immunol* **167**, 2511-21 (2001).
28. Mebius, R.E. et al. The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45+CD4+CD3- cells, as well as macrophages. *J Immunol* **166**, 6593-601 (2001).
29. Yoshida, H. et al. IL-7 receptor alpha+ CD3(-) cells in the embryonic intestine induces the organizing center of Peyer's patches. *Int Immunol* **11**, 643-55 (1999).
30. Fukuyama, S. & Kiyono, H. Neuroregulator RET initiates Peyer's-patch tissue genesis. *Immunity* **26**, 393-5 (2007).
31. Hashi, H. et al. Compartmentalization of Peyer's patch anlagen before lymphocyte entry. *J Immunol* **166**, 3702-9 (2001).
32. Yokota, Y. et al. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature* **397**, 702-6 (1999).
33. Sun, Z. et al. Requirement for RORgamma in thymocyte survival and lymphoid organ development. *Science* **288**, 2369-73 (2000).
34. Finke, D., Acha-Orbea, H., Mattis, A., Lipp, M. & Kraehenbuhl, J. CD4+CD3- cells induce Peyer's patch development: role of alpha4beta1 integrin activation by CXCR5. *Immunity* **17**, 363-73 (2002).
35. Meier, D. et al. Ectopic lymphoid-organ development occurs through interleukin 7-mediated enhanced survival of lymphoid-tissue-inducer cells. *Immunity* **26**, 643-54 (2007).
36. Dougall, W.C. et al. RANK is essential for osteoclast and lymph node development. *Genes Dev* **13**, 2412-24 (1999).
37. Kong, Y.Y. et al. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* **397**, 315-23 (1999).
38. Naito, A. et al. Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells* **4**, 353-62 (1999).
39. Kawaguchi, R. et al. A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. *Science* **315**, 820-5 (2007).
40. Vilhais-Neto, G.C. & Pourquie, O. Retinoic acid. *Curr Biol* **18**, R191-2 (2008).
41. Rask, L. et al. The retinol-binding protein. *Scand J Clin Lab Invest Suppl* **154**, 45-61 (1980).
42. Duester, G. Retinoic acid synthesis and signaling during early organogenesis. *Cell* **134**, 921-31 (2008).
43. Zile, M.H. Vitamin A and embryonic development: an overview. *J Nutr* **128**, 455S-458S (1998).
44. Niederreither, K. & Dolle, P. Retinoic acid in development: towards an integrated view. *Nat Rev Genet* **9**, 541-53 (2008).
45. Sockanathan, S. & Jessell, T.M. Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons. *Cell* **94**, 503-14 (1998).
46. Niederreither, K. et al. The regional pattern of retinoic acid synthesis by RALDH2 is essential for the development of posterior pharyngeal arches and the enteric nervous system. *Development* **130**, 2525-34 (2003).

47. Vermot, J. et al. Retinaldehyde dehydrogenase 2 and Hoxc8 are required in the murine brachial spinal cord for the specification of Lim1+ motoneurons and the correct distribution of Islet1+ motoneurons. *Development* **132**, 1611-21 (2005).
48. Ivanchuk, S.M., Myers, S.M., Eng, C. & Mulligan, L.M. De novo mutation of GDNF, ligand for the RET/GDNFR-alpha receptor complex, in Hirschsprung disease. *Hum Mol Genet* **5**, 2023-6 (1996).
49. Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F. & Pachnis, V. Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* **367**, 380-3 (1994).
50. Edery, P. et al. Mutations of the RET proto-oncogene in Hirschsprung's disease. *Nature* **367**, 378-80 (1994).
51. van de Pavert, S.A. et al. Chemokine CXCL13 is essential for lymph node initiation and is induced by retinoic acid and neuronal stimulation. *Nat Immunol* **10**, 1193-9 (2009).
52. Mucida, D. et al. Retinoic acid can directly promote TGF-beta-mediated Foxp3(+) Treg cell conversion of naive T cells. *Immunity* **30**, 471-2; author reply 472-3 (2009).
53. Hammerschmidt, S.I. et al. Stromal mesenteric lymph node cells are essential for the generation of gut-homing T cells in vivo. *J Exp Med* **205**, 2483-90 (2008).
54. Mebius, R.E. Vitamins in control of lymphocyte migration. *Nat Immunol* **8**, 229-30 (2007).
55. Iwata, M. Retinoic acid production by intestinal dendritic cells and its role in T-cell trafficking. *Semin Immunol* **21**, 8-13 (2009).
56. Mora, J.R. & von Andrian, U.H. Role of retinoic acid in the imprinting of gut-homing IgA-secreting cells. *Semin Immunol* **21**, 28-35 (2009).
57. Veiga-Fernandes, H., Foster, K., Patel, A., Coles, M. & Kioussis, D. Visualisation of lymphoid organ development. *Methods Mol Biol* **616**, 161-79 (2010).
58. Peixoto, A., Monteiro, M., Rocha, B. & Veiga-Fernandes, H. Quantification of multiple gene expression in individual cells. *Genome Res* **14**, 1938-47 (2004).
59. Rossant, J., Zirngibl, R., Cado, D., Shago, M. & Giguere, V. Expression of a retinoic acid response element-hsplacZ transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes Dev* **5**, 1333-44 (1991).
60. Vilhais-Neto, G.C. et al. Rere controls retinoic acid signalling and somite bilateral symmetry. *Nature* **463**, 953-7 (2010).
61. Fu, M. et al. Vitamin A facilitates enteric nervous system precursor migration by reducing Pten accumulation. *Development* **137**, 631-40 (2010).
62. Forster, R. et al. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* **87**, 1037-47 (1996).
63. Ansel, K.M. et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* **406**, 309-14 (2000).
64. Holmes, C. & Stanford, W.L. Concise review: stem cell antigen-1: expression, function, and enigma. *Stem Cells* **25**, 1339-47 (2007).
65. Cacalano, G. et al. GFRalpha1 is an essential receptor component for GDNF in the developing nervous system and kidney. *Neuron* **21**, 53-62 (1998).
66. Moore, M.W. et al. Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76-9 (1996).
67. Vermot, J. et al. Conditional (loxP-flanked) allele for the gene encoding the retinoic acid-synthesizing enzyme retinaldehyde dehydrogenase 2 (RALDH2). *Genesis* **44**, 155-8 (2006).
68. Kioussis, D. & Pachnis, V. Immune and nervous systems: more than just a superficial similarity? *Immunity* **31**, 705-10 (2009).

ANNEXES

I. USED SOLUTIONS

BABB – Benzyl Alcohol and Benzyl Benzoate (Sigma-Aldrich®) in a 1:2 dilution

DMEM supplemented – GIBCO® Dulbecco's Modified Eagle Medium (Invitrogen™) containing 4.5g/L of glucose, pyruvate and glutamine, supplemented with 2% Foetal Bovine Serum (Invitrogen™), 1% Penicillin and Streptomycin (Invitrogen™) and 1% Glutamine (Invitrogen™).

Erythrocyte Lyses Buffer – 144 mM NH4Cl (Sigma) in 17mM Tris HCl (Sigma) solution (pH 7.2)

FACS buffer – PBS containing 2% Foetal Bovine Serum (Invitrogen) and 0,05% Sodium Azide(Sigma).

MeOH – methanol (MeOH from Sigma-Aldrich®) was diluted in PBS-Triton or BABB.

PBS – 10x Phosphate Buffered Saline (pH 7.2) (Invitrogen) was diluted to 1x in sterile Milli Q H_2O.

PBS-Triton 0,15% – Triton X-100 (Sigma-Aldrich®) was diluted in 1x PBS

PFA 4% – Paraformaldehyde (Sigma-Aldrich®) was diluted to 1x PBS

RPMI – GIBCO® Roswell Park Memorial Institute medium (RPMI) (Invitrogen™) containing 4.5g/L of glucose, pyruvate and glutamine, supplemented with 10% Foetal Bovine Serum (FBS) (Invitrogen™), 1% Penicilin and Streptomycin (P/S) (Invitrogen™) ans 1% Glutamin (Invitrogen™).

II. MULTIPLEX PCR

Supplementary table 1. Multiplex Primers. Specific primers used for the first and second PCR. Primers on the top: 5' primers; Primers on the bottom: 3' primers. Reverse transcription reactions were carried out using the 3' primer indicated for each individual gene. Accession numbers correspond to gene sequences obtained at the Ensembl Project Homepage (http://www.ensembl.org).

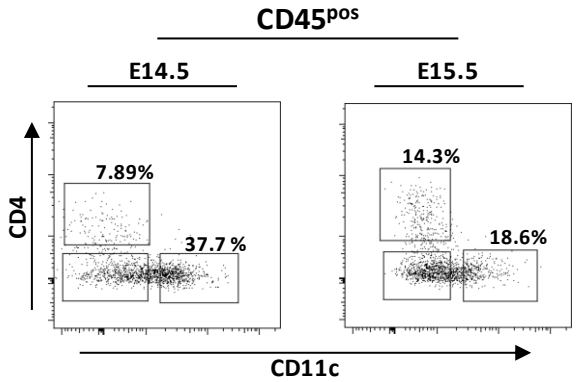
Gene	Ensembl Gene ID	1st PCR Primers	2nd PCR Primers
Cxcl13	ENSMUSG00000023078	5'-AGACTCCGAGCTAAAGGTTG-3' 5'-TCCTCGTGCCAAATGGTTAC-3'	5'-AATGAGGCTCAGCACAGCAA -3' 5'- TCCTCGTGCCAAATGGTTAC-3'
Cxcl12	ENSMUST00000109820	5'-TCCTCTTGCTGTCCAGCTCT-3' 5'-GCTTTCTCCAGGTACTCTTG-3'	5'-AAACCAGTCAGCCTGAGCTA-3' 5'-GCTTTCTCCAGGTACTCTTG-3'
Raldh2	ENSMUSG00000013584	5'-TTCATTGAGCCCACCGTGTT-3' 5'-TGAGTTTGGCTTACGGGAGT-3'	5'-ATGACATGCGGATTGCCAAG-3' 5'- TGAGTTTGGCTTACGGGAGT -3'
Hprt	ENSMUSG00000025630	5'-GCTTTGTATTTGGCTTTTCC-3' 5'-TCCCTGGTTAAGCAGTACAG-3'	5'-GACCTCTCGAAGTGTTGGAT-3' 5'-TCCCTGGTTAAGCAGTACAG-3'

III. GENOTYPING PRIMERS

Supplementary table 2: Genotyping Primers. Specific primers used for the genotyping PCR. Primers the top: 5' primers; Primers at the bottom: 3' primers. For genotyping *Ret* deficient mice the first to pair of primers were used.

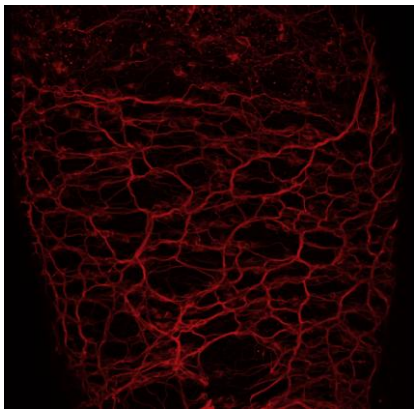
Gene	Primers
Ret WT	5'-TGGGAGAAGGCGAGTTTGGAAA-3' 5'-TTCAGGAACACTGGCTACCATG-3'
Ret null	5'-AGAGGCTATTCGGCTATGACTG-3' 5'-CCTGATCGACAAGACCGGCTTC-3'

IV. HAEMATOPOIETIC POPULATIONS IN THE GUT



Supplementary figure 1. Haematopoietic cell populations in embryonic gut. Representative FACS plots analysis for haematopoietic cell populations at different days of embryonic life in the gut show $CD45^{pos}CD4^{pos}CD11c^{neg}$ Lymphoid Tissue inducer (LTi) and $CD45^{pos}CD4^{neg}CD11c^{pos}$ Lymphoid Tissue initiator (LTin) cells.

V. Neural Crest cell Staining



Supplementary figure 2. **Network of neuronal class III β-tubulin in the gut.** Embryonic gut of E15.5 were immunostained with anti-TUJ1 and analysed by confocal microscopy.

VI. TIME-LAPSES

Supplementary videos of the time-lapses analysis are in the CD-ROM attached to the written document.

Supplementary video 1. **Time-lapse of E15.5 embryonic gut stimulated with BSA.** CD2-GFP embryos were microdissected and then treated with BSA for 24h. In blue, red and green we have examples of cell tracks.

Supplementary video 2. **Time-lapse of E15.5 embryonic gut stimulated with RA.** CD2-GFP embryos were microdissected and then treated with BSA for 24h. In blue, red and green we have examples of cell tracks.

VII. UNITS AND ABBREVIATIONS

Units

µL – microlitre

mL – milliliter

L– litre

µm – micrometer

ng – nanogram

mg – milligram

µM – micromolar

mM – millimolar

Abbreviations

APC: Allophycocyanin

ARTN: Artemin

BABB: Benzyl Alcohol/Benzyl Benzoate

BALT: Bronchus-associated Lymphoid Tissue

BD: Becton Dickinson

BSA: Bovine Serum Albumin

CCR: Chemokine (C-C motif) Receptor

CD: Cluster of Differentiation

cDNA: complementary Desoxyribonucleic Acid

c-fms: Colony Stimulating Factor 1

CT: Cycle Threshold

CXCL: Chemokine (C-X-C motif) Ligand

CXCR: Chemokine (C-X-C motif) Receptor

DC: Dendritic Cell

DMEM: Dulbecco's Modified Eagle Medium

E: Embryonic day

FACS: Fluorescence Activated Cell Sorting

FBS: Foetal Bovine Serum

Foxp3: Forkhead box P3

GALT: Gut-associated Lymphoid Tissue

GDNF: Glial Derived Neurotrophic Factor

GFP: Green Fluorescent Protein

Gfrα: Glial derived Neurothrophic Factor Family Receptor alfa

HCl: Hydrochloric acid

HPRT: Hypoxanthine-guanine phosphoribosyltransferase

ICAM-1: Inter-Cellular Adhesion Molecule 1

IgA: Immunoglobulin A

IGC: Instituto Gulbenkian de Ciência

IL: Interleukin

KCl: Potassium Chloride

LN: Lymph Nodes

LTi: Lymphoid Tissue Inducer

LTin: Lymphoid Tissue Initiator

LTo: Lymphoid Tissue Organizer

LTα1β2: Lymphotoxin alpha 1, beta 2

LTβR: Lymphotoxin beta Receptor

MAdCAM-1: Mucosal Addressin Cell Adhesion Molecule-1

MALT: Mucosa-associated Lymphoid Tissue

MeOH: Methanol

$MgCl_2$: Magnesium Chloride

MuLV: Moloney Murine Leukemia Virus

ODF: Osteoclast Differentiation Factor

ODFR: Osteoclast Differentiation Factor Receptor

OPGL: Osteoprotegerin Ligand

PCR: Polymerase Chain Reaction

PP: Peyer's Patches

RA: Retinoic Acid

RALDH: Retinaldehyde Dehydrogenase

RANK: Receptor Activator for Nuclear Factor κ B

RANKL: Receptor Activator for Nuclear Factor κ B Ligand

RAR: Retinoic Acid Receptor

RARE: Retinoic Acid Response Element

RBP4: Retinol Binding Protein 4

Ret: Rearranged during Transfection

RNA: Ribonucleic Acid

RPMI: Roswell Park Memorial Institute

RT-PCR: Real-time Polymerase Chain Reaction

SLOs: Secondary Lymphoid Organs

TNF: Tumor Necrosis Factor

TNFSF11: Tumor Necrosis Factor (ligand) Superfamily, member 11

TRAF6: TNF Receptor-associated Factor 6

TRANCE: TNF-related Activation-induced Cytokine

TRANCE-R: TNF-related Activation-induced Cytokine Receptor

TSLP: Thymic Stromal Lymphopoietin

VCAM-1: Vascular Cell Adhesion Molecule-1

LIST OF FIGURES

LIST OF TABLES

LIST OF VIDEOS